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A Quarterly Journal of Zoölogical Research

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CONTENTS OF VOLUME XVIII

NUMBER 1

Behavior of Melanoblasts Derived from the Coelomic Lining in Interbreed Grafts of Wing Skin . . . . . *Mary E. Rawles* 1

Some Group Effects on the Rate of Cleavage and Early Development of Frog Eggs (*Rana pipiens*) . . . . . *Ruth Merwin* 16

Ebony: A Gene Affecting the Body Color and Fecundity of *Tribolium confusum* Duval *Thomas Park, Benson Ginsburg, and Shirley Horwitz* 35

The Golden Hamster (*Cricetus auratus*): Care, Breeding, and Growth *Charlotte R. Bond* 52

Studies on Metabolism of *Taenia taeniaeformis* . . . . . *James H. Wilmoth* 60

Some Factors Affecting Excystment in the Ciliate *Tillina magna* . . *C. D. Beers* 80

New Books in Physiological Zoölogy 100
Franz Schrader, Mitosis, *C. W. Metz.*

NUMBER 2

The Effects of Atmospheric Humidity on Animal Life . . . . . *Daniel Ludwig* 103

Studies on the Biochemistry of *Tetrahymena*. III. Strain Differences *George W. Kidder and Virginia C. Dewey* 136

A Simple Method for Division-Rate Determination in *Paramecium* . *Arthur C. Giese* 158

Physiological Studies on Hibernation in the Chipmunk *Alvalyn E. Woodward and John M. Condrin* 162

Effects of X-rays upon Flecking in the Domestic Pigeon *Leon J. Cole and Harold E. Finley* 167

Some Aspects of the Dominance-Subordination Relationship in the Fish *Platypoecilus maculatus* . . . . . *James Conger Braddock* 176

Some Effects of Thiamin on the Winning of Social Contracts in Mice *Elizabeth A. Beeman and W. C. Allee* 195

New Books in Physiological Zoölogy 222
Hugh Davson and James Frederic Danielli, The Permeability of Natural Membranes, *A. K. Parpart*

NUMBER 3

Ultraviolet Radiations and Life . . . . . *Arthur C. Giese* 223

Oxidation-Reduction Patterns in Ciliate Protozoa. . . . . *C. M. Child* 250

Relations between the Reproductive Cycle and the Pituitary Gland in the Snake *Thamnophis radix* . . . . . *Edwin S. Cieslak* 299

New Books in Physiological Zoölogy 330
Forest Ray Moulton (ed.), The Chemistry and Physiology of Hormones, *Robert Gaunt.*—Arnold Gesell, in collaboration with Catherine S. Amatruda, The Embryology of Behavior, *William F. Windle*

NUMBER 4

Experimental Alteration of Growth Rate in Chimaeric Feathers of Breast-Saddle Origin in the Brown Leghorn Capon . . . . . *Hsi Wang* 335

Mating Behavior and the Social Hierarchy in Small Flocks of White Leghorns *A. M. Guhl, N. E. Collias, and W. C. Allee* 365

Bisexual Mating Behavior in the Male Rat: Effects of Castration and Hormone Administration . . . . . *Frank A. Beach* 390

Influence of the Stimuli Provided by the Male Cichlid Fish, *Tilapia macrocephala*, on the Spawning Frequency of the Female . . . . . *Lester R. Aronson* 403

Antigenic Relationships in the Genus *Tetrahymena* *George W. Kidder, C. A. Stuart, Virginia G. McGann, and Virginia C. Dewey* 415

Mineral Deficiency as a Factor Limiting Growth of the Ciliate *Tetrahymena geleii* *R. P. Hall and W. B. Cosgrove* 425

New Books in Physiological Zoölogy 431
S. C. Brooks and Matilda Moldenhauer Brooks, The Permeability of Living Cells, *M. H. Jacobs.*—Hans Schaefer, Elektrophysiologie, Vol. I: Allegemeine Elektrophysiologie; Vol. II: Spezielle Elektrophysiologie, *Frank A. Brown, Jr.*

Index to Volume XVIII 437

158

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Volume XVIII JANUARY 1945 *Number 1*

BEHAVIOR OF MELANOBLASTS DERIVED FROM THE COELOMIC LINING IN INTERBREED GRAFTS OF WING SKIN

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Department of Biology, Johns Hopkins University

In an earlier series of experiments (Willier and Rawles, 1940) it was observed that melanoblasts from an embryo of one breed of fowl, introduced into the base of the wing bud of an embryo of a different breed before the entrance of its own neural crest cells, were unable to set up a permanent source of donor precursor pigment cells in the host wing skin. It will be recalled that, as a rule, the melanoblasts migrated extensively from the site of implantation and invaded the developing feather germs of the entire host wing and often adjacent regions, with the result that the down plumage of the affected area showed donor coloration. As time went on and later generations of contour feathers appeared, the donor color gradually disappeared from successively regenerating feathers until finally the whole region took on the normal color of the host. On the basis of the experiments of Foulks (1943), which have shown that a feather germ must receive a fresh supply of melanoblasts from the surrounding dermis for each successive generation of feathers, it would appear that, as the donor melanoblasts were used up during the process of feather pigmentation, host melanoblasts moved into the region to replace them.

It has been demonstrated, by grafting an area of wing skin, experimentally deprived of its normal source of pigment cells, to the back of young host chicks of the same or of a different breed (Rawles, 1942, 1944), that the melanoblasts from the surrounding host skin will migrate readily into the graft. Under these conditions the invading host melanoblasts have always produced the pattern of their specific breed in the successive generations of feathers developing from the graft, irrespective of breed or sex of either donor or host. In contrast to these results, Danforth and Foster (1929) and Danforth (1929) found that, as a rule, no migration of host melanoblasts into the graft occurred when an area of normal back skin was grafted in a similar manner between two newly hatched chicks of different breeds. The grafts under such conditions continued to regenerate donor-colored feathers (except for a few mosaics appearing occasionally at the margins). From these results it is inferred that melanoblasts do not enter an area of skin which contains at the time

[1] The writer wishes to express her appreciation to Professors B. H. Willier and C. H. Danforth for a critical reading of the manuscript and to Mr. John Spurbeck for the drawings and photographs.

of grafting a sufficient quantity of melanoblasts to provide for the pigmentation of its future generations of feathers, or, in other words, a normal complement of pigment cells. By the time of hatching, ordinary skin has acquired this full, normal complement of melanoblasts. That a similar complement can also be acquired after the hatching period has been clearly demonstrated by the pigment-free skin grafts already referred to.

In view of the results obtained from skin grafting, the question arises as to whether the disappearance of the donor coloration from the regenerating feathers of our original experiments is correlated with the introduction of an inadequate quantity of donor melanoblasts. In these experiments it was observed that the extent and the duration of the donor coloration were determined, to a large degree, by such factors as the size of the implant, the age of the donor embryo, and the region from which the isolate was taken, all of which would influence the quantity of melanoblasts introduced. If the observed "wearing-out" of the donor pigmentation effect were directly correlated with the introduction of an inadequate quantity of melanoblasts, as the evidence would seem to indicate, then one should be able to prolong the donor coloration or, perhaps, even make it permanent by increasing the quantity of melanoblasts introduced into a given area of skin (the wing bud, for example). For doing this, the intracoelomic grafting method seemed to offer certain definite advantages over that originally employed without affecting in any way the interbreed setup. In the first place, by removing the wing bud from the donor embryo before its own neural crest cells (source of pigment cells) entered and by placing it in the coelom of a host embryo (approximately the same age) of a genetically pigmented breed, a continuous and abundant supply of pigment-forming cells would be available for a long period of time, i.e., from approximately the third day until hatching. (The experiments of Eastlick, 1939, have demonstrated that a graft growing in the coelom will take up pigment-forming cells from a pigmented host if it becomes properly attached.) It seems reasonable to suppose that the chances would thus be increased for introducing a larger number of precursor pigment cells than could be introduced when only one "dose" was put into the wing bud in the form of a tiny piece of donor tissue. In the second place, grafting to the coelom would eliminate entirely the possibility of any competition between two types of melanoblasts (donor and host) during embryonic development, since only one type (host) would have access to the graft during its period of growth and differentiation in the coelom. At hatching, the fully developed wing skin with its complement of melanophores acquired from the parietal lining of the host coelom could be regrafted easily to the back region of a newly hatched chick of any desired breed, and the duration of the pigmentation effect could be followed in the series of regenerating juvenile and adult feathers.

The results of this method of approach and their bearing on the phenomenon of the replacement of foreign melanoblasts during feather regeneration will be presented in the following pages.

PROCEDURE

The experimental procedure for introducing melanoblasts of one breed of fowl into the skin and developing feather germs of a different breed is illustrated in Figure 1. Two transplantation techniques were employed: (1) transplantation to the embryonic coelom and (2) skin grafting at hatching. Since both

have been used extensively and described previously, no detailed description is necessary. (For technical details concerning the first, see Hamburger, 1939; for the second, see Danforth and Foster, 1929; and for a combination of the two, similar to that used in the present experiments, see Rawles, 1944.)

White Leghorn embryos (66–75 hours) were used exclusively for donors. Barred Plymouth Rock and Rhode Island Red embryos (66–71 hours) were used for the first hosts; and newly hatched chicks of White Leghorn, Barred Plymouth Rock, and Silver Campine breeds were used for the second hosts. Briefly, the procedure was the following: the wing bud or the prospective wing bud (somatopleure adjacent to somites 16–20) was removed from the right side of the White Leghorn donor embryo, before the entrance of neural crest cells, and transferred to the

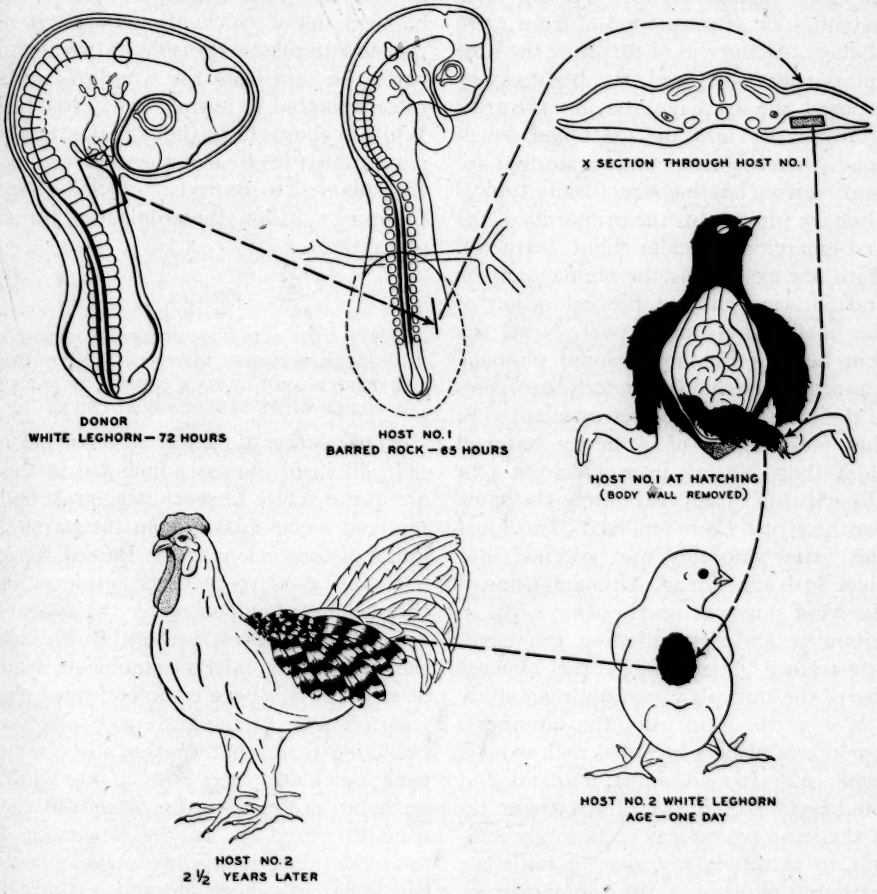


FIG. 1.—A series of diagrams illustrating the method of introducing Barred Rock melanoblasts from the parietal peritoneum into White Leghorn wing skin, its retransplantation at hatching to the back of a newly hatched White Leghorn host, and the subsequent plumage development.

coelom of a Barred Rock or a Rhode Island Red host embryo (host No. 1) through a slit in the somatopleure made just anterior to the prospective hind-limb region. In this very favorable site the graft was allowed to develop until hatching, i.e., for a period of from 17 to 18 days. At the end of this time the host embryo was sacrificed, the body cavity exposed, the sex diagnosed, and the graft removed. By now the graft had developed, in most cases, into a normal-appearing wing bearing structurally typical plumage (down plus the primordia of the first-emerging juvenile flight feathers). With few exceptions the plumage of the grafted wing showed the coloration of the host entirely or in part. Next, the skin bearing the host-colored plumage was dissected from the underlying tissues of the wing (graft) and retransplanted to the back region of a newly hatched chick (host No. 2), from which an area of back skin of approximately the same size had just been removed. The graft was fitted smoothly and stitched into place with silk thread. Although some of the wing skin was nearly always lost in trimming and straightening the edges preparatory to stitching, the average size of the transplant was approximately 1 × 2–3 cm. Skin from the ulnoradial region was always included and at least some from the adjoining humeral and hand regions. Skin from the extreme tip of the hand region was exceedingly difficult to manipulate, owing to both the restricted width and the comparatively large size and rigidity of the primordia of the primary flight feathers, and hence it was often discarded. Occasionally, however, it was possible to remove and transplant practically the entire area of wing skin.

The White Leghorn donor embryos were usually saved and allowed to continue their development. For various reasons the eggs at the time these experiments were in progress were particularly nonviable. However, in 8 of the 20 cases upon which this report is based the donors lived long enough for their sex to be diagnosed, but in only 1 case was the hatched donor chick strong enough to risk an autoplastic transplantation. In 16 cases the skin from the wing graft was retransplanted to normal newly hatched White Leghorn chicks (homoplastic transplantation); in 3 cases the skin was retransplanted to Barred Rock and Silver Campine chicks (heteroplastic transplantation).

RESULTS

SERIES 1. THE RETRANSPLANTATION OF WHITE LEGHORN WING SKIN, DIFFERENTIATED IN THE BARRED PLYMOUTH ROCK COELOM, TO NEWLY HATCHED WHITE LEGHORN HOST CHICKS

Pigmentation of the intracoelomic graft.—In all 14 of the cases included in this group the White Leghorn wing graft had received melanoblasts from the parietal lining of the coelom of the Barred Rock host during its 17–18 days' residence in the coelom, as evidenced by the appearance of black down feathers. In 8 cases the invasion of host melanoblasts may be said to have been complete, since the wing plumage of the graft was indistinguishable from that of the host's own wing (all black except for a few white feathers characteristically present at the wing tip [Fig. 1]). In the remaining 6 cases the migration of host melanoblasts had apparently been blocked or limited to some extent, since the down plumage of the graft was only partially pigmented—the distal portion of the wing mostly white, the proximal portion either all black or a mixture of black and white. In 2 extreme cases the entire wing plumage was predominantly white with only a sprinkling of black down feathers among the white of the upper wing region.

Whether a graft grown in the coelom of a pigmented host becomes wholly or only partly pigmented appears to depend upon how completely the skin proper unites with the parietal lining of the coelom containing the host melanoblasts. In all cases in which the graft-plumage showed normal host pigmentation, the edges of the skin at the base of the humerus (where attachment always was made) were observed to be continuous with the parietal peritoneum of the host, establishing a relatively broad connection between graft and host tissues. But, in those cases in which the graft-plumage was only partly host colored, it was observed that the skin had receded to a varying extent along the humerus, away from the point of attachment of the base of the humerus to the inner body wall (somatopleure) of the host and thus only indirectly and somewhat narrowly connected with the parietal lining containing the melanoblasts. A similar relationship between graft attachment and the degree of host pigmentation exhibited by the graft was pointed out by Eastlick (1939). The attachment of the graft to the mesenteries of the intestine of the host rather than to the somatopleure, a condition which he observed rather frequently, was only rarely found in the present experiments, in which a definite effort was made to orient the graft in such a way that it would become attached to the somatopleure.

The fate of the grafted skin.—Of the 14 newly hatched White Leghorn chicks (hosts No. 2) which received transplants of skin from the aforementioned intracoelomic wing grafts, 1 died too early (within the first week) to provide any data regarding the ultimate fate of the transplant. Of the total number of 13 which survived, 3 were failures, in that the area of grafted skin became necrotic and was shed within 3–5 weeks after grafting without having produced any contour feathers. In the remaining 10 cases the grafts became definitely established. But in 2 of these 10 cases signs of regression began to appear in the grafted area while the feathers were still in the pinfeather stage (6–8 weeks); consequently, these 2 cases contributed little to the present study. However, in 8 cases the graft has produced well-developed, structurally normal contour feathers of the wing type. Three of the individuals have maintained their grafts until the present time (over 2½ years), and the grafted area of skin has continued to produce wing feathers in a perfectly normal manner. In these 3 cases the grafts have become permanent without any doubt. For a discussion of host-graft relationships in similar skin-transplantation experiments and for a description of the various forms the graft failures take during regression the reader is referred to a recent communication (Rawles, 1944).

The regenerated feathers of the grafted skin.—Without exception the feathers produced by the grafted skin were of the wing type—flight feathers (remiges) and coverts—and characteristic of the portion of the wing from which the skin was taken (Pl. I, Figs. 2 and 3).

In the cases in which the down was uniformly and intensely pigmented (black) the contour feathers which followed in due time were always pigmented and showed the characteristic black-and-white barring found in homologous feathers of the Barred Rock host that furnished the melanoblasts. One graft of the wholly pigmented type which has persisted until the present time (over 2½ years) and which has completed successfully two natural molts of adult plumage is shown in Figure 2. Barred feathers have been regenerated continuously and in normal succession without having

shown any sign whatsoever of pigment depletion or the "wearing-out" phenomenon in a single feather. As many as 5 or 6 generations of adult feathers have been obtained from certain of the flight-feather germs of this graft as a result of plucking the feather when it was completely matured rather than waiting for the natural molt. In this particular case there can be no doubt that the Barred Rock host melanoblasts which migrated from the parietal lining into the White Leghorn skin and feathers of the wing graft prior to hatching have succeeded in setting up a permanent "reservoir" or source of pigment-forming cells for supplying the future generations of feathers arising from the grafted skin. Whether the 5 other grafts in this category (down completely pigmented at the time of retransplantation to White Leghorn host No. 2) would have behaved likewise had they persisted longer than 2–3+months remains, of course, an open issue. However, it should be noted that, up until the time necrosis set in, pigmented feathers of the juvenile and the adult plumage showing a perfectly normal barred pattern were being regenerated.

Grafts only partly host colored at the time of retransplantation have produced both white and pigmented (barred) contour feathers. In general, the white feathers developed from the regions covered with white down, the pigmented from the black regions; but various exceptions have occurred. Sometimes pigmented pinfeathers have appeared, here and there, where the down was white. Usually in such cases the skin was found to be rather heavily pigmented. Regions of mixed (grayish-black and white) down have nearly always produced all-white contour feathers. In 2 cases in which the grafts remained permanently and have been followed into the second year, 1 case regenerated only white feathers, despite the fact that the plumage at the time of retransplantation contained a few black down feathers scattered among the white. In the other case (No. 283) the more interesting of the two, the upper (proximal) third of the wing skin at the time it was retransplanted bore black down, while the remaining portion bore chiefly white down. The pigmented portion gave rise to juvenile and adult feathers completely pigmented and exhibiting the typical barred pattern of the Barred Rock host which furnished the melanoblasts. The lower white portion produced white feathers with the exception of one barred primary flight feather, which at the completion of its growth was replaced by one entirely white.

For a while it looked as if the Barred Rock melanoblasts had set up a permanent source of pigment-forming cells in the proximal portion of this graft. But, as time passed, white feathers and barred-and-white mosaic feathers began to appear, gradually replacing those that were all barred as they were either shed naturally or plucked at maturity. At the present time (second year) most of the regenerating feathers are white. Split preparations of several samples have revealed the presence of White Leghorn melanophores, showing that the role of pigmentation is being taken over by the second host. There seems little doubt that ultimately the plumage of the graft will be entirely hostlike (white).

Whenever the graft regenerated pigmented feathers, either temporarily or permanently, the type of barring displayed has followed, without exception, that characteristic of the sex of the Barred Rock individual (host No. 1) which furnished the melanophores (e.g., the greater width and intensity of the black bars of the female, as contrasted with the male). Neither the sex of the donor nor the sex of the second host had

any perceptible effect on the quality of the barring (see Table 1). Cases Nos. 258 and 260 are of particular interest in demonstrating the control of barred-pattern dimorphism by the melanophore. It will be observed from the tabulation that in case No. 258 the area of wing skin with its prospective feather germs came originally from a male White Leghorn embryo (donor). During its period of development in the coelom of a Barred Plymouth Rock female (host No. 1), the transplant received melanoblasts, genetically female, from the coelomic lining of the host. Although the retransplantation at hatching was made to a male White Leghorn (host No. 2), the barred pattern produced in the contour feathers of the graft was that of a typical Barred Plymouth Rock female. Since the only female components of the entire combination were the melanophores, it is evident that the pattern was under the direct control of the female melanophores and not affected by the male constitution of either the feather germs of the donor or the hormones of the second host. Similarly, in case No. 260 the male melanophores which entered the graft while it was in the coelom of a Barred Plymouth Rock male host produced a normal male barred pattern when regrafted to the back of a female White Leghorn host. The fact that the sex of the donor was not known in this case and in some of the others listed in the table is not highly important, in view of the results of previous skin-transplantation experiments (Rawles, 1944, pp. 175–76), which demonstrated clearly that the tone and quality of the plumage pattern produced by immigrant Barred Plymouth Rock melanophores are not altered by the sex or the breed of the donor which furnishes the feather germs. (For a discussion of the genotypic control of feather color patterns the reader is referred to Willier and Rawles, 1944*a* and *b*.)

One interesting feature of this series of retransplantations has been the appearance of a barred pigment pattern in a few of the saddle feathers of the White

TABLE 1*

SUMMARY OF DATA PERTAINING TO BREED, SEX, AND PIGMENTATION OF THE WING-SKIN GRAFTS WHICH PRODUCE TYPICAL WING FEATHERS

Case No.	Donor Embryo (66–75 Hours) (Source of Wing Bud)		Host No. 1. Embryo (66–71 Hours) (Wing Bud in Coelom)			Host No. 2. Young Chick (½–7 Days) (Recipient of Wing Skin from Intracoelomic Wing Graft of Host No. 1)		
	Breed	Sex	Breed	Sex	Color of Down of Wing Graft	Breed	Sex	Color Pattern of Regenerated Feathers of Grafted Wing Skin
Ser. 1:								
257....	W.L.	Unknown	B.P.R.	Female	Black	W.L.	Female	B.P.R.—female
258....	W.L.	Male	B.P.R.	Female	Black	W.L.	Male	B.P.R.—female
260....	W.L.	Unknown	B.P.R.	Male	Black	W.L.	Female	B.P.R.—male
262....	W.L.	Female	B.P.R.	Male	Black	W.L.	Male	B.P.R.—male
263†...	W.L.	Unknown	B.P.R.	Male	Black	W.L.	Male	B.P.R.—male
267†...	W.L.	Male	B.P.R.	Male	White chiefly; trace of black	W.L.	Female	W.L.
270....	W.L.	Unknown	B.P.R.	Female	White chiefly; trace of black	W.L.	Female	W.L.
283†...	W.L.	Unknown	B.P.R.	Female	Part black; part white	W.L.	Female	B.R.—female; W.L.; mosaics
Ser. 2:								
287†...	W.L.	Female	R.I. Red	Female	Part reddish buff; part white	W.L. (orig. donor)	Female	R.I. Red; W.L.; mosaics
429....	W.L.	Female	R.I. Red	Female	Part reddish buff; part white	B.P.R.	Male	R.I. Red; B.P R.—male; mosaics
461†...	W.L.	Male	R.I. Red	Female	White chiefly; trace of reddish buff	S. Campine	Female	Silver Campine

* W.L. = White Leghorn; B.P.R. = Barred Plymouth Rock; R.I. Red = Rhode Island Red.
† Grafts persisted.

Leghorn hosts growing adjacent to the wing feathers of the graft. These exceptional, pigmented host feathers have been of two kinds: (1) completely barred feathers and (2) color mosaics made up of varying proportions of barred and solid white areas. Such feathers have shown up in 5 of the 8 cases listed in Table 1, in which the whole graft or a part (case No. 283) was intensely pigmented (black) at the time the wing skin was regrafted to the back of the White Leghorn hosts. The type of barring (male or female) produced has always corresponded exactly with that exhibited by the wing feathers of the graft, showing unmistakably that the melanoblasts responsible for the pigmentation have come into the host back feathers from the grafted piece of skin.

Successive generations from the affected host feather germs may or may not be colored. For example, 3 hosts, each of which produced several pigmented saddle feathers during the time that the grafted skin persisted, all regenerated pure-white feathers (host colored) after the graft (source of the barred melanophores) was shed. In another individual (case No. 283), in which the graft persisted but the feathers gradually lost their color over a period of nearly 2 years, 10 host feather germs bordering the graft regenerated barred or partly barred (mosaic) feathers, but during the time when only barred feathers were being regenerated by the graft. Case No. 263, the only one in which the grafted skin and the coloration of the regenerated feathers have both persisted permanently, produced no exceptional barred host feathers for a long time—until the completion of the second natural molt of adult plumage. After this molt 2 barred saddle feathers appeared, one on either side next to the graft. The replacement feathers from each of these follicles have now practically completed their development and are typically barred.

SERIES 2. THE RETRANSPLANTATION OF WHITE LEGHORN WING SKIN, DIFFERENTIATED IN THE RHODE ISLAND RED COELOM, TO NEWLY HATCHED CHICKS OF WHITE LEGHORN, BARRED ROCK, AND SILVER CAMPINE BREEDS

Six cases are included in this series. In 3 cases the retransplantation was made to White Leghorns, one of which was the original donor; in 1 case, to a Barred Rock; and in 2 cases to Silver Campine chicks. Two of the chicks—1 White Leghorn and 1 Campine—died a week afterward, and another (White Leghorn) shed its graft before contour feathers developed. Therefore, only 3 of the original 6 grafts actually contributed to the present study. These 3, listed in Table 1, developed typical wing contour feathers. Two of the cases (Nos. 287 and 461) have retained their grafts permanently, i.e., until the present time ($2\frac{1}{2}$ years). The results of this series are similar to those already described for Series 1. During its period of development in the coelom the graft received pigment cells from the coelomic lining of the host, as evidenced by the appearance of down feathers with the reddish-buff coloration characteristic of the plumage of the newly hatched chicks of the Rhode Island Red (host) breed. As in the preceding series, some of the grafts were entirely host colored, when removed from the coelom; others were only partly host colored, with a varying proportion of unpigmented (white) plumage. As chance would have it, the 3 grafts which persisted after being regrafted to the second hosts represented the partly pigmented type and consequently have not retained the acquired red host coloration indefinitely in the regenerated feathers.

Case No. 287 of this series is very much like case No. 283 of Series 1. At the time when the retransplantation was

made to the newly hatched White Leghorn back (incidentally, this individual was the original donor; hence the transplantation was autoplastic), the skin bore reddish-buff down and unpigmented (white) down in approximately equal proportions. Also, the primordia of most of the flight feathers, when examined from the reverse side of the dissected skin prior to stitching, showed both red and black pigment, characteristic of the Rhode Island Red (host) breed. Of 16 flight feathers which regenerated without delay, 10 were completely pigmented, 6 were unpigmented. The pigmented feathers all showed the characteristic Rhode Island Red wing pattern (predominantly red with varying amounts of solid black coloration). In addition to the flight feathers, numerous red covert feathers developed from the region where the red down predominated; white coverts, from the predominantly white region. While many of the feather germs continued to reproduce adult feathers completely pigmented for 3 or 4 feather generations (induced by plucking the feathers from selected follicles when fully matured), others after the first generation reproduced white or partly white mosaic feathers. By the end of the first year all of the pigmented feathers had become replaced or were in the process of being replaced by white feathers like those of the host.

In the other two cases (Nos. 429 and 461) of this series the migration of pigment cells from the coelomic lining into the graft had been even more limited than in the preceding case. Case No. 461 showed only a trace of red in the plumage at the time of retransplantation to the Campine host. None of the regenerated feathers were red. The vast majority showed the characteristic black-and-white barred Campine host pattern and have continued to reproduce this pattern. Several unpigmented, structurally normal contour feathers have appeared among those showing the typical Campine pattern. For some reason these few feather germs did not become invaded by the Campine host melanoblasts. (An account of such exceptional all-white feathers in skin grafts may be found in a recent paper by Rawles, 1944, pp. 177, 180). Case No. 429 regenerated a few wing feathers with the characteristic Rhode Island Red color pattern and several mosaic red and barred wing feathers up until the time the graft was shed at the end of 3+ months. The majority of the regenerating feathers, however, showed the typical barred pattern of the Barred Plymouth Rock host. Where the second host represents a pigmented breed, the readiness with which host melanoblasts enter pigment-free regions of the graft after contact is established with the surrounding host skin is very clearly demonstrated (cf. Rawles, 1944).

DISCUSSION

From the present experiments it would appear that the quantity of pigment cells which migrate into the grafted wing bud from the coelomic lining of the host determines whether the induced pigmentation pattern is able to persist indefinitely in the feathers regenerating from the grafted skin. While no method has been devised for determining the exact number of pigment-forming cells which migrate into the graft from the host embryo, nevertheless we can judge the relative number of pigment cells which have invaded each of the grafts of the same experimental series by comparing the extent and intensity of their coloration with the normal wing of the host at the time the wing grafts are removed from the coelom. To illustrate, let us examine 3 individual birds (cases Nos. 263, 283, and 267) which have retained their skin

grafts permanently and which are representative of the different types of results obtained from the same experimental procedure. When the wing graft of case No. 263 was removed from the Barred Rock coelom, its plumage was as completely and intensely pigmented (black) as that of the Barred Rock host wing. The broad and complete union of the skin of the graft with the parietal peritoneum of the coelomic wall had permitted a free migration of host pigment cells into the graft. In the next case (No. 283) the migration of pigment cells from the host embryo had been limited by a less complete union of the wing skin of the graft with the parietal lining of the host; therefore, instead of being completely pigmented, like case No. 263 and the host, this wing graft became only partially pigmented, i.e., partly black, partly white (pigment-free). Between the predominantly black and the predominantly white regions there was a scattering of lightly pigmented, grayish-black down. In the third case (No. 267) the Barred Rock host pigment cells had been even more restricted in their migration than in case No. 283. The wing skin had receded along the humerus, away from the point of attachment, and was, therefore, only indirectly connected with the parietal lining of the host. As a result, this wing was predominantly white, i.e., pigment-free, with only a few lightly colored, grayish-black down feathers scattered among the white in a small area near the place of attachment. Clearly, then, these 3 grafts show a definite gradation in the relative number of host pigment cells received from the parietal lining. When the pigmentation of the regenerating wing feathers is followed in these same cases after the retransplantation of their skin to the back of newly hatched host chicks, we find that the fully pigmented type is the only one in which the acquired barred host coloration has persisted indefinitely. While the partly colored type produced several successive generations of barred wing feathers from the pigmented portion of the graft, the role of pigmentation was finally taken over by the melanoblasts which migrated into the graft from the second host (White Leghorn). The most lightly pigmented of all (No. 267) regenerated no pigmented feathers but all white feathers, like the Leghorn host. Thus, we see in these grafts evidence of a distinct relationship between the relative number of Barred Rock pigment cells introduced and the duration of the Barred Rock pattern. Where graft attachment has permitted a full and unlimited migration of host pigment cells, no replacement of the grafted menaloblasts has occurred.

When these three types of results are compared with the results of other transplantation methods, we immediately see striking similarities regarding the behavior of melanoblasts. The fully pigmented type of transplant has behaved essentially like the skin transplants of Danforth (1929), in that the melanoblasts carried over with the area of grafted skin have continued to reproduce their own specific color pattern in the regenerated feathers (except occasionally at the line of union of graft and host skin). In both, the grafted area of skin had received by the time of hatching a complement of melanoblasts sufficient to provide the feather germs it contained with a permanent source of pigment cells. Whether the complement was acquired by allowing the skin to remain in its normal position until hatching, as in Danforth's experiments, or by early isolation and subsequent attachment to the coelomic wall of a pigmented host of another breed, as in the present experiments, in no way affected the final results in regard to the persistence of the

original pigmentation pattern in the regenerating feathers of the graft. From such results it would, therefore, appear that pigment cells do not readily enter an area of skin which already contains an adequate quantity of melanophores. As a rule, each melanophore type occupied a circumscribed region of the host—the grafted melanoblasts remaining in the grafted skin, the host melanoblasts remaining in the host skin. However, occasionally at or near the line of union of host and graft skin a migration of the two types of melanoblasts did occur, giving rise to host feathers showing the coloration of the graft, and color mosaics, i.e., feathers showing the color pattern of both donor and host. It was pointed out earlier (p. 8) that in 5 of the 8 cases of Series 1 of the present experiments, in which the entire graft or a part (No. 283) was fully pigmented at the time the wing skin was regrafted, structurally normal host saddle feathers developed which were either completely barred, like the feathers of the graft, or were color mosaics made up of varying proportions of barred (graft-colored) and white (host-colored) portions. The design of the present experiments is decisive for determining the origin of mosaic feathers, since the wing feathers of the graft and the back feathers of the host are morphologically distinct. Without any exception the mosaics have been either back (saddle) feathers or wing feathers, never structural chimaerae.

The wing grafts which during their period of development in the coelom of the pigmented hosts received either no pigment cells, or at most only a few, have behaved after transplantation essentially like the melanophore-free wing-skin grafts reported previously (Rawles, 1942, 1944); that is, melanoblasts from the surrounding host skin were not blocked or held in check but migrated readily into the pigment-free skin and feather germs of the graft, where they continued indefinitely to reproduce their own specific type of pattern in the developing feathers. Case No. 461 of this group has been of particular interest in demonstrating for the first time the formation of the typical barred wing pattern of the Silver Campine breed (host No. 2) in grafted wing feathers of White Leghorn origin (for a detailed account of the barring pattern of this individual, see Nickerson, 1944). No "wearing-out" or disappearance of the pigmentation pattern has ever occurred when melanoblasts of one breed were introduced into the developing feathers of another breed by this method of grafting (see Rawles, 1944). Whether the host melanoblasts establish a "reservoir" of stem cells in the grafted skin or whether they continue to migrate in from the surrounding host skin as each successive generation of feathers develops is not known, although it is probable, considering Danforth's results with normal skin, that a source is established within the graft itself. However, the fact remains that the source of pigment cells becomes inexhaustible once the graft establishes connection with the host skin.

Between the two extremes of fully pigmented transplants which received no pigment cells from the surrounding host skin after retransplantation and the pigment-free or almost pigment-free transplants which received their entire supply of melanoblasts from the surrounding host skin, we have a third, more or less in-between, type of wing graft which became only partly pigmented during the period of development in the coelom. After retransplantation the pigment cells of such grafts were gradually replaced by those of the second host, but not until many of the feather germs had produced from 1 to 3 or more successive

generations of adult feathers showing the characteristic coloration of the breed from which the melanoblasts came originally (Barred Rock or Rhode Island Red). This gradual replacement of the grafted melanoblasts by those of the host was strikingly similar to that observed in our original experiments, in which melanoblasts were transplanted directly to the wing bud with a tiny piece of tissue. The fact that the coloration was prolonged in the present cases indicates that the supply of grafted melanoblasts was more adequate.

The above-mentioned transition in feather coloration can be explained on the basis of the findings of Foulks (1943). He has shown that even normal feather germs (Barred Rock) do not contain a permanent supply of melanophore stem cells but receive from the surrounding dermis a fresh complement for supplying each successive feather generation. Therefore, it would appear that, as the melanoblast content of the grafted area of skin is gradually exhausted during the process of feather pigmentation, other melanoblasts from the host—the only ones available—move in and take over the role of pigmentation in the graft. Since the renewal of the melanophore content of feather germs is not peculiar to experimental conditions but recurs in normal feather regeneration, it seems unnecessary to postulate some reaction on the part of the host to account for the elimination of the grafted pigment cells. The fact, further, that the replacement of the grafted pigment cells by those of the host occurs so gradually and over such a long period of time—3 or 4 months to well over a year in the present cases—furnishes strong evidence against any kind of incompatibility reaction.

While we cannot ignore the theoretical possibility of the existence of a melanophore-host incompatibility between breeds, the evidence is far from conclusive that such an incompatibility does actually exist. If we examine the data from other transplantation series, we find that, whenever melanoblasts of one breed or species have been introduced into the feather germs of another breed by the method of direct grafting to the embryonic wing bud, the grafted melanoblasts have reproduced their own specific color pattern in the regenerating host wing feathers for a temporary period only; replacement of the donor coloration by that of the host has always followed, and relatively early (usually during the emergence of the juvenile plumage). Even when the donor melanoblasts came from such distantly related birds as the robin, crow, red-winged blackbird, etc., the results have been consistently the same. In view of the large numbers of cases dealt with in these experiments (over 200 individuals have been followed through their various plumage changes), it is difficult to explain the invariable results on an incompatibility basis alone. According to the laws of chance, we should occasionally expect to find two individuals in which the melanoblasts and the feather germs were compatible, yet none was ever found. In contrast to the above series, we have the melanophore-free wing-skin grafts in which melanoblasts of one breed migrated into the pigment-free feather germs of another breed. Under such conditions the foreign melanoblasts continued to reproduce their own specific color pattern in the wing feathers developing from the graft, irrespective of the breed origin of the grafted area of skin. No "wearing-out" or disappearance of the foreign pigment cells ever occurred. That we should have, in every case, complete compatibility between the melanoblasts and the feather germs of this series of experiments and complete

incompatibility without exception in the preceding series, where many of the same breeds were dealt with, seems quite unlikely. It has already been pointed out in the preceding pages that the principal experimental results can be drawn together on the basis of the quantity of melanophores introduced. It is of interest to note in this connection that the recent work of Nickerson (1944) on the formation of barred patterns in feathers indicates that a certain quantity of melanophores is necessary to bring the intensity of pigmentation to a minimum level required for the production of bars.

Many questions arise that cannot be answered on the basis of our present information concerning melanophore behavior. For example, in the cases in which the number of foreign melanoblasts introduced into an area of skin is limited by the method, as in our original experiments, and the partly colored cases of the present experiments, it is not clear why some of the melanoblasts are not held over in an undifferentiated stem-cell stage to provide a permanent source of pigmentation for a small number of feather germs, instead of all continuing to the end-point of differentiation (pigment deposition in the feather epidermal cells). In this connection it is of interest to note that even prospective neural crest cells taken from the medullary plate region of early blastoderms likewise failed to establish a permanent source of pigmentation when grafted to the wing bud (see Rawles, 1940). So far, there are no data available which give us any precise information concerning the nature of the factors which control so effectively the melanophore content of an area of skin. The attraction of host melanoblasts to grafts of pigment-free skin, their almost complete exclusion from normally pigmented skin grafts, and their gradual admittance into partially pigmented skin grafts—all lead us to suspect that some definite numerical proportion or ratio of pigment cells to skin cells must be established and maintained during the process of feather pigmentation.

The evidence which has accumulated in recent years from interbreed transplantation experiments in fowl is in complete agreement in showing that the melanoblasts themselves directly determine not only pigment production but pattern organization as well. While there is some evidence that the epidermis of a particular feather tract may modify the details of the general breed pattern produced by the melanophore, there is no evidence whatsoever that the persistence of the initial pattern is in any way affected by the breed origin of the epidermis. More than once, melanoblasts of one breed have produced their specific color pattern permanently in the epidermis of the developing feathers of an entirely different breed (Nos. 263 and 461 from the present series and 3 cases from the 1944 series). Many others undoubtedly would have continued to reproduce their initial pattern had the grafted skin persisted, since at least 2 successive generations of feathers were produced—often more—before the graft was shed, without any evidence of pigment replacement. However, the 5 cases cited above are more than sufficient to demonstrate conclusively that the epidermis and the pigment cells do not have to come from the same breed-source, either donor or host, in order for the initial coloration to be maintained in successive generations of feathers, as suggested by Wang (1944, pp. 117–18).

An examination of the feathers which have developed at or near the line of union of graft (wing) and host (back) skin has furnished no evidence whatsoever of a joint origin of the feather papillae. It has already been pointed out

that structurally normal saddle feathers have been observed which showed the coloration of the graft entirely or in part; and, similarly, structurally normal wing feathers have been observed which showed the coloration of the host entirely or in part. If the epidermis of a feather tract determines the specificity of the resulting feather, as the studies of Lillie and Wang (1941, 1943) have shown, then the very fact that the mosaic feathers which have developed have been either saddle or wing feathers, never saddle-wing chimaerae, is proof enough that the graft follicular epidermis has not entered into combination with that of the host. On the basis of such clear-cut results with wing-skin grafts, it is extremely improbable, contrary to the interpretation of Wang (1943, p. 346), that the graft follicular epidermis has combined with that of the host to form "composite papillae" in Danforth's experiments in which back skin was similarly grafted to the back region of a newly hatched host chick. The mosaic feathers described by Danforth (1929, 1935, 1937) are essentially like those of the present experiments and can be accounted for entirely on the basis of melanoblast migration. Correctly speaking, the mosaic feathers which occasionally arise when areas of newly hatched skin are grafted between breeds are color mosaics, not structural mosaics. For a discussion of the melanophore composition of mosaic feathers occurring in skin grafts, as well as those occurring naturally in hybrid breeds, the reader is referred to Foulks (1943, pp. 373–78).

Finally, the demonstration given by the present experiments that melanoblasts of the coelomic wall can produce the typical color and pattern of their breed if introduced into the skin and feather germs of another breed is worthy of emphasis. During the course of embryonic development, these deeper-lying melanoblasts would never ordinarily reach the skin with its developing feather germs. The point which is most striking is not that these melanoblasts are capable of developing pigment—that they normally produce pigment granules is well known to anyone who has examined the parietal peritoneum of chicks of pigmented breeds—but that they are potentially capable, once they get into feather germs, of producing in the developing feathers all the intricacies of pattern characteristic of their breed, including sexual dimorphism—a very nice example, it would seem, of the interplay of hereditary and environmental factors in the development of melanophores.

SUMMARY

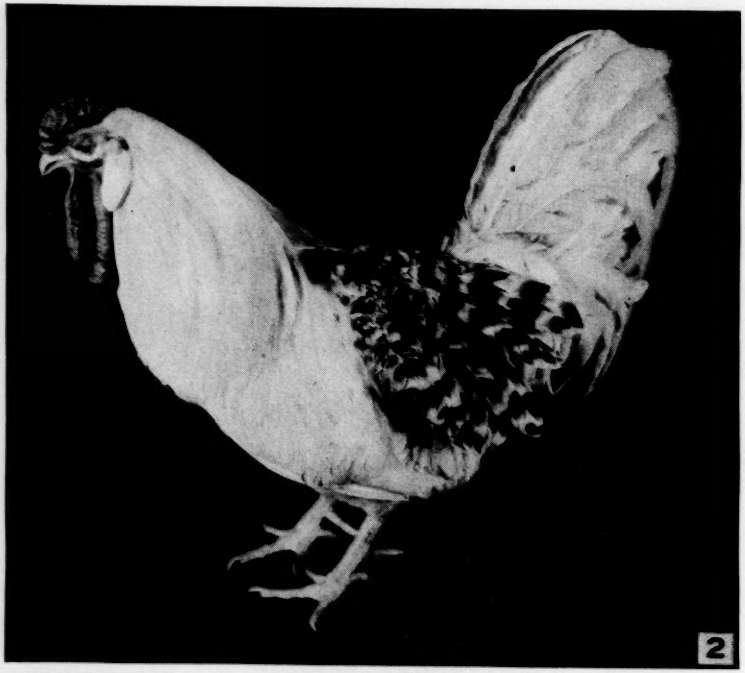
1. The present investigation is concerned with the relationship between the quantity of foreign melanoblasts introduced into an area of grafted skin and the persistence of the initial pigmentation pattern in successive generations of feathers.

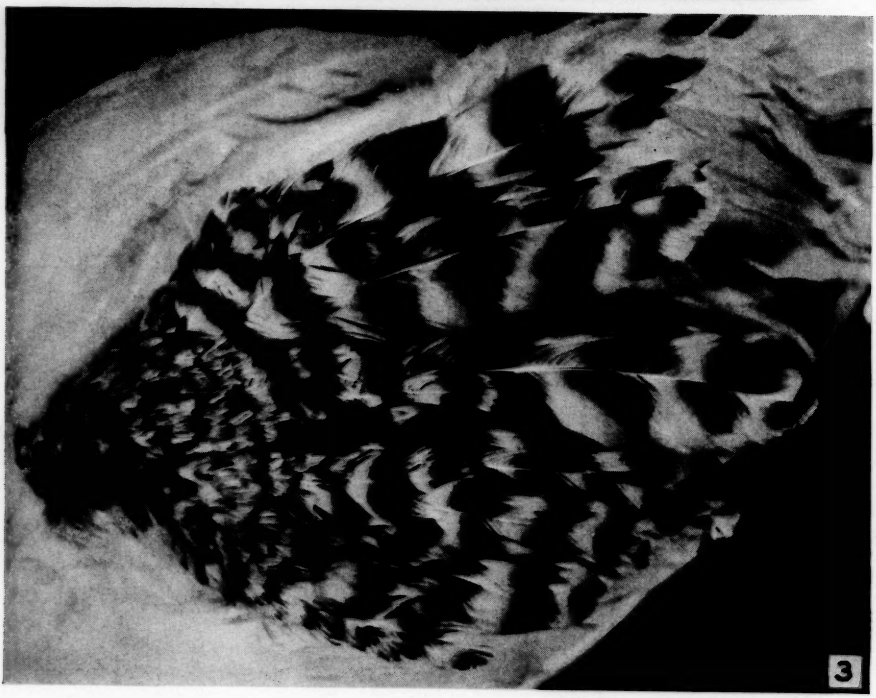
2. By removing the right wing bud from White Leghorn embryos (before the entrance of neural crest cells) and allowing it to develop until hatching in the coelom of a host embryo of a pigmented breed (Barred Plymouth Rock or Rhode Island Red) foreign pigment cells have been introduced into White Leghorn wing skin and feather germs.

3. The relative number of pigment cells which migrated into the wing graft from the parietal peritoneum of the host during its period of development in the coelom was determined by comparing the coloration of the down plumage of the graft with that of the normal host wing at the time the graft was removed from the coelom.

4. With respect to the degree of host pigmentation exhibited by the down plumage of the wing, three types of

PLATE I





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grafts were obtained: (*a*) fully pigmented—indistinguishable from the wing of the host; (*b*) partly pigmented—partly host colored, partly white (pigment-free); (*c*) predominantly unpigmented—chiefly white with only a trace of host color. This gradation in host coloration was found to be directly correlated with the completeness of union of the skin of the graft with the parietal peritoneum of the host (source of pigment cells).

5. By regrafting the wing skin to the back region of newly hatched host chicks (White Leghorn, Barred Rock, Silver Campine) the development of the acquired pigmentation pattern and its persistence in the successive generations of feathers which developed from the graft was followed.

6. Grafts which were entirely host colored at the time of retransplantation have regenerated wing feathers continuously which have shown the characteristic color pattern of the first host (Barred Rock or Rhode Island Red) which furnished the melanoblasts. Grafts partly host colored at the time of retransplantation have regenerated wing feathers with the color pattern of the first host for a temporary period only. Replacement of the grafted melanoblasts by those migrating into the graft from the second host took place gradually. After the first year most of the feathers regenerating from the graft have displayed the normal color pattern of the second host. Grafts which were pigment-free or almost pigment-free at the time of retransplantation have produced wing feathers with the color pattern of the second host only. From these results it would appear that the persistence of the initial pattern in the regenerating feathers of the graft is determined by the quantity of pigment cells initially introduced. Where graft attachment permitted a free migration of host pigment cells no replacement of the grafted melanoblasts by those of the second host has occurred.

7. The present experiments have demonstrated that a full, normal complement of melanoblasts can be acquired from the parietal lining of the coelom of a host of a pigmented breed. Although these deeper-lying melanoblasts ordinarily do not reach the skin, they are potentially capable, once they get into feather germs, of producing all the intricacies of pattern characteristic of their breed, including barred-pattern dimorphism.

8. The design of the present experiments is decisive for determining the origin of feathers of mosaic coloration, since the wing feathers of the graft and the back feathers of the host are morphologically distinct. Without exception the mosaics which have developed have been either back (saddle) feathers or wing feathers, never structural chimerae.

PLATE I

FIG. 2.—Case No. 263. White Leghorn male (host No. 2), 2½ years after receiving a graft of wing skin of White Leghorn origin invaded by Barred Rock pigment cells during its period of development in the coelom of a Barred Plymouth Rock male (host No. 1). The graft has continued to regenerate wing feathers completely and typically barred. (Overdeveloped comb removed during second year.)

FIG. 3.—A close-up of graft of Fig. 2. Note typical wing plumage and characteristic Barred Plymouth Rock male pattern.

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SOME GROUP EFFECTS ON THE RATE OF CLEAVAGE AND EARLY DEVELOPMENT OF FROG EGGS (RANA PIPIENS)[1]

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AN OPTIMAL density has been demonstrated for speeding cleavage and early development of sea-urchin eggs. Five species have been included in the work of the following investigators: Frank and Kurepina (1930), Maxia (1933), Allee and Evans (1937*a* and *b*), and Sugiyama (1938). The question arose as to whether other eggs would show similar effects, and the frog's egg was selected for further study. Yolk-plug and later stages, as well as early cleavages, were observed in various population densities. The present inquiry is part of an extensive study concerning the effects of population density on organisms that is being conducted by Professor Allee and his associates.

[1] The writer wishes to acknowledge her gratefulness to Professor W. C. Allee for his interest in and advice on this problem.

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MATERIALS AND METHODS

Frogs (*Rana pipiens*) were secured from Wisconsin and kept at approximately 6° C. in glazed earthenware jars, lined with moist paper. They were used within 5 or 6 weeks after being received. Frogs with eggs unovulated could not be secured from outside sources after the first of May, but females put at 6° about

the first of May still produced eggs showing a high percentage of fertility on June 1. At the time of injection frogs were placed in a constant-temperature room that was held at approximately 16°. The methods of Rugh (1934), with modifications, were used in obtaining and fertilizing eggs. Fresh, finely ground-up frog pituitary was suspended in water at a concentration of one female or two male pituitaries to 1 cc. of water. During the winter each female received two subcutaneous injections of two pituitaries each, 24 hours apart. In the spring one pituitary at each injection was sufficient to induce ovulation. From 36 to 48 hours after the first injection the eggs could be obtained by stripping the female. Even though fertilization would not take place or the fertility was low the first day that they were available, the unstripped eggs often showed a high percentage of fertility 2–5 days later and for a period of 1 week thereafter. The physiology of the eggs did not change so long as the fertility remained high—at least relative to the characters studied in this paper. Females in which eggs had been ovulated were kept at 6° until about 15–30 minutes before the eggs were stripped, when they were placed at 16°. Only data from experiments in which the cleavage was 65 per cent or over are reported in this paper, except for some experiments described in Table 2, where the percentage of fertility is recorded. In many cases the fertility was very nearly 100 per cent.

A sperm suspension was prepared by macerating the testes from uninjected male frogs in approximately 8 cc. of water. The suspension was freed of all pieces of testis tissue and allowed to stand at least 20 minutes at 16° before use. It was diluted or used directly, depending on the concentration. Such a suspension gave high fertility for 2 days if kept at 6°.

During the first half of the work, testes from two or three males were frequently combined in making up a sperm suspension; later, larger males were used, and one was sufficient. Often one experiment included several females, but the eggs from different ones were kept separate throughout the experiment and were suitably distributed between experimentals and controls. Eggs from only one female were used in any one paired comparison. No marked, consistent individual differences showed up in an analysis of cases where two or three females were used in the same experiments.

In most cases the eggs were stripped into a clean glass dish, usually a Petri dish. In a few cases they were stripped onto paraffin paper. The sperm suspension was poured on and left, as a rule, for 10 minutes, although it could be left for from a few seconds to 30 minutes. The eggs were drained, rinsed twice, and then allowed to stand well covered with water. The jelly swelled to such an extent that, when the eggs were crowded, they had to be loosened from the bottom. In some experiments the eggs were separated, by means of needles, into groups or single eggs and placed in other dishes. This could not be done with safety until approximately 1 hour after fertilization. Between 5 and 30 seconds after the sperm suspension is added, frog eggs become susceptible to shaking or touching and will not cleave if disturbed. At the end of 30 minutes they can be loosened from the bottom but will not stand being cut apart. Cutting can be done without preventing cleavage of some or all of the eggs only after the eggs have turned. Because of this period when the eggs cannot be manipulated, the methods for cleavage study used in this paper can be separated into two groups: those in which the eggs were bunched or scattered at the time of fertilization, before the

sensitive period; and those bunched or scattered 1 hour after fertilization, following the sensitive period. As will be seen, the results differ in the two cases. Differences in methods will be pointed out for each series of experiments when the results are presented.

Three different types of dishes were used. In many of the experiments the eggs were placed in jelly glasses that held 125 cc. of water. The diameter of the bottom was $2\frac{1}{2}$ inches, and 135 eggs could be placed in a single layer over the bottom. Glass-stoppered Erlenmeyer flasks of 100-cc. capacity were also used, as were liter flasks with rubber stoppers.

Eggs killed in the cleavage stages were killed during either the second, third, fourth, or fifth cleavages; and they were under experimental conditions 300–600 minutes. Eggs left until the yolk plug had formed and the blastopore was closing were under experimental conditions 40–60 hours. Scattered eggs were at least approximately 1 cm. apart. Bunched eggs either were not cut or were shoved together as closely as possible.

The time for killing was determined for yolk-plug stages by direct observation. For cleavages a separate dish was placed under the same conditions as the rest of the eggs and handled as little as possible. At killing time, most of the water was decanted, and the eggs were poured through a funnel into a 100 per cent formalin solution, diluting the solution to approximately 12 per cent; this solution, in turn, was diluted further, to approximately 3 per cent formalin, within an hour. The amount of 100 per cent formalin used depended on the number of eggs to be killed. Bunched eggs were usually in a layer one egg thick; and, since they presented less surface for the killing agent to penetrate than single eggs did, they might have been killed more slowly. Evidence that such a difference is not significant in the present work is presented later. Eggs used in paired comparisons were killed at the same time; or, if there was a few seconds' lapse between killing, the scattered eggs were always killed last. For instance, in some comparisons scattered eggs from four dishes were considered as one member of a paired comparison, and eggs bunched in one dish were the other member. It was possible to tip three dishes of eggs into formalin at one time, one bunched and two scattered; then, as soon as possible, but actually a few seconds later, the other two scattered lots were killed.

An effort was made to kill the cleaving eggs when 50 per cent of the control eggs had gone through the cleavage in question—either the second, third, fourth, or fifth cleavage. Results of the counts on fixed eggs are recorded in percentage cleaved for any one cleavage studied. Eggs were recorded as cleaved when the first indication of a cleavage furrow appeared. There was no selection of data near 50 per cent cleavage. Except in one or two paired comparisons, data were not used where either the experimental or the control eggs had not begun or had already completed cleavage. Under the conditions of these experiments, after cleavage started, approximately 4 per cent more eggs began cleavage each minute until all were cleaved. This estimation is based on observation of the second cleavage.

Yolk plugs were measured with an ocular micrometer which magnifies one and one-quarter times. Two diameters at right angles were obtained in micrometer units, and for comparative purposes the product was used as a relative area, without calculating the actual area. Since the eggs were killed when the product of the diameters was in some cases over 100 and in others under 10 and since the change in size per hour is much greater when the

plugs are large, the differences in size of plug between experimental and control lots of eggs were changed into time intervals, for comparative purposes. Eggs in a wide range of yolk-plug stages were killed at hourly intervals and a graph made from the results (see Fig. 1). From this graph one can determine for any size yolk plug the expected change per hour and thus approximately translate the measured difference between the bunched and scattered eggs into hours. Too few counts were made to give an accurate curve. Also, the long-range changes in temperature in the experimental room prevent these translations into time from being anything more than a rough approximation.

The entire experimental procedure after transferring the male and female frogs from the 6° room was carried out in a large constant-temperature room having a floor space of 7 × 8 feet and a ceiling 10 feet high. The temperature was maintained by circulating conditioned air. Dishes containing eggs to be used in paired comparisons were placed as close together as possible. Flasks were stoppered, jelly glasses were covered with glass squares, and a whole tableful of covered jelly glasses was again covered with a box to prevent differences in temperature due to eddies in the circulating air. As a rule, the temperature did not vary during any one experiment, judging from observations of a thermometer calibrated to 1° C., but ranged from 12° to 17° over long periods. Experimental dishes were kept covered, as far as possible, except in one set of experiments, which will be pointed out later.

Synthetic pond water was used exclusively in the experiments. It was made up in 20-liter lots by adding the following chemicals of reagent quality: 50 mg. of $MgSO_4 \cdot 7H_2O$, 100 mg. of $CaCl_2 \cdot 4H_2O$, 50 mg. of $NaNO_3$, and 50 mg. of K_2SO_4 to a liter of distilled water. The distilled water was known to be of good quality from tests of conductivity and from use in other experiments in the laboratory (Allee, Finkel, and Hoskins, 1940). The prepared "pond" water was brought to equilibrium with the air at 16° by bubbling through it, for at least 12 hours, compressed air that had been washed in distilled water. A lower CO_2 content was obtained by the continuous aeration of the water during the experiment by means of air passing through a train containing NaOH, followed by distilled water. The eggs were protected from the agitation resulting from the aeration process. If a higher CO_2 concentration was desired, "pond" water saturated with CO_2 at 6° was added. The CO_2 was obtained from the reaction of HCl and $CaCO_3$ and then washed through distilled water. The concentrations desired required from 1 to 10 cc. per liter of CO_2-saturated "pond" water. Carbon dioxide tensions were measured by the Seyler (1894) method, using a 0.01 N

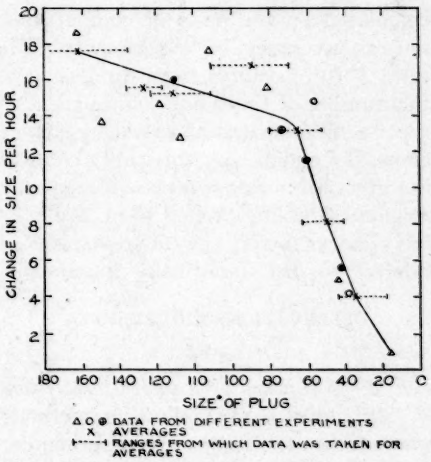

FIG. 1.—Changes, in size per hour, of yolk plugs of various sizes. Size is in micrometer units and is a relative area. See text, p. 18.

solution of Na_2CO_3 in titrating. The CO_2 values so obtained may be a little low, according to Papp (1943).

The data were analyzed statistically by the paired comparison method of "Student" (1925). Each comparison consisted of one experimental and one control set of eggs. In the accompanying tables the average of these comparisons per experiment is recorded, as well as the average of all comparisons, regardless of the number of experiments from which they were obtained. The *P*-values are calculated on the basis of comparisons, not as averages of experiments. The term *P* (probability) is an expression of the number of times one could expect as great a difference as observed by chance alone. If *P* equals 0.05, there are 5 chances in 100 of obtaining as great a difference by random sampling. When there are 5 or less chances in 100, the difference is considered to be statistically significant.

OBSERVATIONS AND RESULTS

CLEAVAGE

a) Eggs bunched or scattered at the time of fertilization.—The following methods were used in the first set of experiments, designed to determine whether grouped eggs cleave more rapidly than do isolated ones. The same methods were repeated another year to confirm the results. Ten to several hundred eggs were stripped into a Petri dish; the sperm suspension was poured on; and the dish was shaken so that some eggs were scattered and some were left in small or larger bunches one layer thick over the bottom. After 20 minutes the sperm suspension was removed; the eggs were washed and water was poured on; then the eggs were loosened from the bottom. About 1 hour after fertilization the eggs turned, and the scattered and the grouped eggs were removed and arranged in separate jelly glasses. An important point to note here is that the eggs which at the time of fertilization became separated from the others, or those at the edges of the bunches, were selected and used as isolated eggs, while the bunched eggs were usually obtained by cutting up larger groups into smaller ones. Thus the eggs were really bunched or scattered from the time of fertilization but were placed in separate dishes an hour afterward.

It can be seen from Table 1 that under these conditions there is a significant tendency for bunched eggs to cleave more rapidly. The data from work done in two different years are tabulated on different lines. The comparisons for the first year are divided on the basis of size of group. This is of interest in the light of later experiments. The larger and smaller groups of eggs are not significantly ahead of the scattered eggs, although a trend is indicated. Since in these experiments the isolated eggs were manipulated more than the bunched ones, the bunched eggs in 2 of the 1943 experiments were partially cut apart; that is, they were not cut enough so that they became separated. This did not prevent the bunched eggs from cleaving sooner. Other experiments on this question of the effect of cutting will be discussed later. A total of 93 comparisons from 12 experiments listed in Table 1 show that, on the average, the bunched eggs were ahead of the scattered ones by 8.2 per cent cleavage. This difference has a *P*-value of 0.0000414. In these experiments, as in the next set, the bunched, uncut eggs did not present so much surface to the killing agent and might not have been killed so quickly as single eggs. To test this possibility, one experiment was carried out in which the bunched eggs were cut before killing, and these were also ahead of the single eggs.

Seventeen more experiments were per-

formed in which the eggs were isolated or bunched from the time of fertilization. The results are given in Table 2. The eggs, some isolated and some grouped, were left in the dish in which they were fertilized—not removed to other dishes with isolated and bunched eggs separated. Two methods were used in getting the eggs scattered or bunched. Results from the two methods were alike and have been combined in Table 2. Either the eggs were shoved around just prior to or immediately after fertilization, before they became sensitive to disturbance, to get them into the desired scattered or grouped arrangements, or the eggs were only shaken and never touched. Comparisons in which the fertility of the eggs is below 65 per cent are treated separately. Also, comparisons based on only 1 or 2 scattered eggs are not included in the first analysis but are placed in separate lines; they include eggs with both high and low fertility. As in the first set of experiments, there was a tendency for bunched eggs to cleave sooner. The difference is statistically significant in all cases for bunches that range in size between 7 and 21 eggs. Bunches larger in size did not in any case cleave significantly faster than scattered eggs, although a tendency in that direction was present. Small bunches of 2–6 eggs showed a significantly faster cleavage for high plus low fertility comparisons but not for high fertility comparisons alone or for comparisons based on only 1 or 2 scattered eggs. These observations, together with those in Table 1, indicate an optimal-sized bunch of 7–20 for an increase in rate of cleavage. The larger bunches were consistently not significantly ahead. The smaller bunches were not so consistent, but results indicate that eggs in these small bunches cleave less rapidly than do those in bunches of the indicated optimal size.

TABLE 1

DIFFERENCE IN THE RATE OF CLEAVAGE BETWEEN EGGS BUNCHED AND EGGS SCATTERED AT THE TIME OF FERTILIZATION AND THEN PLACED IN SEPARATE DISHES 1 HOUR AFTER FERTILIZATION

Date	No. in Bunch	No. of Expts.	No. of Eggs Used in Comparisons		No. of Comparisons	No. of Comparisons in Which Bunched *minus* Scattered Is in Percentage Cleaved			Bunched *minus* Scattered in Percentage Cleaved		*P*-Value for Comparisons
			Scattered	Bunched		Over +0.6	+0.5 to −0.5	Over −0.6	Av. of Expts.	Av. of Comparisons	
1943.....	4–6	6	694	1,673	14	9	2	3	+ 1.2	+ 5.6	0.2516
	7–20				43	28	4	11	+ 7.0	+ 7.1	0.0164
	21–63				22	11	2	9	+ 8.6	+ 5.6	0.1246
1944.....	6–19	6	174	268	14	10	0	4	+18.1	+16.3	0.0058

If all the comparisons between eggs bunched or scattered at the time of fertilization and not touched thereafter (all those in Table 2) are combined, we find 146 comparisons, with the bunched eggs 10.6 per cent ahead; the *P*-value for the difference is 0.0000962.

Throughout the experiments reported in this paper, frequently the eggs isolated at the time of fertilization had a low fertility, compared to the grouped ones in the same dish. Fertility in this connection means that the eggs did not cleave; however, they may have been fertilized. The fertility of the bunched

TABLE 2

DIFFERENCE IN THE RATE OF CLEAVAGE BETWEEN EGGS BUNCHED AND EGGS SCATTERED AT THE TIME OF FERTILIZATION AND LEFT IN THE SAME DISH TOGETHER

Date	No. of Expts.	No. of Eggs Used in Comparisons		No. of Comparisons	No. of Comparisons in Which Bunched *minus* Scattered Is in Percentage Cleaved			Bunched *minus* Scattered in Percentage Cleaved						*P*-Value of Comparisons
								Av. of Expts.			Av. of Comparisons			
								Size of Bunch						
		Scattered	Bunched		Over +0.6	+0.5 to −0.5	Over −0.6	2–6	7–21	22–63	2–6	7–21	22–63	
		Fertility above 65 per Cent												
1943 and 1944	9	129	135	21	13	0	8	+ 7.6	.....	.....	+11.0	.....	.....	0.1046
	15	340	839	44	28	2	14	.....	+14.0	.....	.....	+10.5	.....	0.0278
	7	132	381	14	7	0	7	.....	.....	+ 4.4	.....	.....	+ 5.0	0.3844
	Fertility of Bunched Eggs, above 65 per Cent; That of Scattered Eggs, below 65 per Cent													
1943 and 1944	3	38	38	7	5	0	2	+14.0	.....	.....	+16.0	.....	.....	0.0908
	4	56	193	11	8	1	2	.....	+21.0	.....	.....	+20.0	.....	0.0158
	5	51	244	7	5	0	2	.....	.....	+10.6	.....	.....	+15.0	0.2754
	Combination of Above Data for Eggs with High and Low Fertility													
1943 and 1944	10	167	173	28	18	0	10	+ 8.6	.....	.....	+12.3	.....	.....	0.0300
	19	396	1,031	55	36	3	16	.....	+16.0	.....	.....	+12.6	.....	0.0027
	12	183	625	21	12	0	9	.....	.....	+ 7.0	.....	.....	+ 8.6	0.1252
	Comparisons Based on One or Two Scattered Eggs, Regardless of Fertility													
1943 and 1944	10	25	68	17	7	4	6	.....	.....	.....	− 0.3	.....	.....	0.6
	8	23	224	16	9	1	6	.....	.....	.....	.....	+17.0	.....	0.0298
	9	12	272	9	5	0	3	.....	.....	.....	.....	.....	+8.1	0.5652

TABLE 3

DIFFERENCE IN PERCENTAGE FERTILE BETWEEN THE BUNCHED AND SCATTERED EGGS FOR THE EXPERIMENTS SUMMARIZED IN TABLE 2

From Each Experiment Extremes in Fertility of Eggs for Comparisons Are Selected and Averaged				Limit of Percentage Fertile below Which No Comparisons Used	
Highest Percentage Fertile		Lowest Percentage Fertile			
Bunched	Scattered	Bunched	Scattered	Bunched	Scattered
98	90	86	76	65	65
93	55	81	40	65	5

and scattered eggs has been analyzed for the experiments shown in Table 2, in an effort to determine whether the fertility is related to the rate of cleavage. See Table 3, where two points can be noted: first, that the scattered eggs were always lower in fertility than the bunched eggs, a condition which may or may not have been the result of the greater mechanical disturbance of single eggs, compared with that of bunched ones, at the time of fertilization; and, second, that when the fertility of bunched eggs dropped 5 per cent between the so-called "high" and the "low" fertility experiments the fertility of the scattered eggs was lowered by 35 per cent. The data in Table 2 indicate that there may be a greater difference in cleavage rate between the bunched and scattered eggs in the lower-fertility comparisons. This suggests that lower fertility is associated with slower cleavage, in which case the greater difference in fertility of the bunched and scattered eggs should correlate with a greater difference in time cleavage. For comparable sets of eggs a difference of 1 between the amount of acceleration at high and at low fertility is not significant; t equals 0.1. For another set of eggs a difference of 16 has a t-value of 1.7 and P equals 0.08913. Although this later P approaches significance, it is based on only 5 cases at the lower fertility. The question needs further investigation.

b) Eggs not bunched and scattered until 1 hour after fertilization.—In the next series of experiments eggs were fertilized in a Petri dish and were kept, as far as possible, in one large group one layer thick. After they turned, about 1 hour after fertilization, the eggs were cut apart, and those which had not turned were removed. The eggs were mixed up and counted at random into jelly glasses In some dishes they were scattered, and in others they were pushed together in a group. The results are given in Table 4. Eggs in bunches of 5 cleaved slightly sooner, on the average, than did accompanying lots of 5 isolated ones. Despite a large number of comparisons, the difference is not statistically significant. The conditions for this particular set of comparisons differ from those for all the others, since the dishes were not covered and since they contained only 25 cc. of water instead of 125 cc. In the remaining experiments summarized in Table 4 a group of 20 eggs gave a slightly earlier appearance of the first few sets of cleav-

TABLE 4

DIFFERENCE IN RATE OF CLEAVAGE BETWEEN EGGS THAT WERE CUT APART 1 HOUR AFTER FERTILIZATION AND THEN SHOVED INTO BUNCHES AND THOSE CUT APART AND THEN ISOLATED

No. of Eggs per Dish		No. of Expts.	No. of Eggs Used in Comparisons		No. of Comparisons	No. of Comparisons in Which Bunched *minus* Scattered Is in Percentage Cleaved			Bunched *minus* Scattered in Percentage Cleaved		*P*-Value for Comparisons
Grouped	Scattered		Grouped	Scattered		Over +0.6	+0.5 to −0.5	Over −0.6	Av. of Expts.	Av. of Comparisons	
5	5	7	460	460	82	46	5	31	+2.1	+3.0	0.2713
10	5	10	910	910	91	39	1	51	−1.7	−1.8	0.3681
20	5	13	1,560	1,560	79	48	3	28	+1.4	+3.2	0.0574
40	5	9	1,020	960	48	23	2	23	+0.4	0.0	.....
10	5	9	260	390	26	11	0	15	−4.8	−5.0	0.1016
40	5	11	1,080	405	27	9	1	17	−4.6	−5.2	0.0456

age furrows when compared with a similar number of scattered eggs. The observed difference approaches statistical significance. A group of 10 was slowed insignificantly, while a group of 40 was significantly retarded. This fits roughly the results obtained with eggs that were isolated or bunched from the time of fertilization, except that here cleavage in the smallest and largest bunches tested was actually retarded, as compared with the scattered eggs. In the instances summarized in Tables 1 and 2 the bunched eggs cleaved more rapidly than did the scattered eggs but seemed relatively retarded, as compared with egg populations that were intermediate in size. Thus, in all these cases the optimal population was intermediate in size during early cleavage.

Since in the experiments described in Table 1 the bunched eggs in most cases were not cut apart while in those described in Table 4 they were cut and then shoved together, it was thought desirable to find out if cutting eggs apart 1 hour after fertilization had any effect on the rate of cleavage. Experiments were set up in which the eggs were treated as in the experiments described in Table 4 except that all eggs were not mixed up; rather, one large bunch was cut from the rest, and then it was cut in half and the two halves placed in separate jelly glasses. One of the halves was cut and the eggs scattered, and one of the halves was bunched. Half of the bunched eggs were left without cutting, and half were cut apart and then shoved together. The cut and uncut bunches both show an insignificant deviation in rate of cleavage from the scattered eggs, thus indicating that cutting had no effect on the rate of cleavage. One would expect that the bunched eggs might have been ahead of the scattered ones, on the basis of the work reported in Table 4. Perhaps the reason they are not is because there is too much deviation from the indicated optimal-sized bunch of 20 eggs.

TABLE 5

DIFFERENCE IN THE RATE OF CLEAVAGE BETWEEN BUNCHED EGGS WHICH HAVE NEVER BEEN CUT APART AND SCATTERED EGGS AND THAT BETWEEN BUNCHED EGGS WHICH HAVE BEEN CUT APART AND THEN SHOVED TOGETHER AND SCATTERED EGGS

No. of Eggs per Bunch	No. of Expts.	Treatment of Bunched Eggs	No. of Eggs Used		No. of Comparisons	No. of Comparisons in Which Bunched *minus* Scattered Is in Percentage Cleaved			Bunched *minus* Scattered in Percentage Cleaved		*P*-Value for Comparisons
			Scattered	Bunched		Over −0.6	+0.5 to −0.5	Over −0.6	Av. of Expts.	Av. of Comparisons	
6–23.....	8	Cut	289	343	30	15	1	14	+0.5	+2.0	0.1204
15–32.....	9	Uncut	660	766	46	22	0	24	−0.6	+0.6	.....

A small set of experiments was carried out in which the rate of cleavage of eggs in dense populations was compared with that in sparse populations. One hundred and thirty-five eggs covered the bottom of a jelly glass, and in some cases the eggs were in two or three layers. The data are summarized in Table 6. None of the differences have statistical significance. The observed differences are small; so in order to determine whether they are significant many comparisons are needed, and at this point too few are available to come to any final conclusion.

The experiments involving eggs iso-

lated or bunched an hour after fertilization were all performed in 1944. Nine of the experiments in which eggs were bunched or isolated from fertilization and the eggs not touched (see Table 2) were also done in 1944. Nevertheless, it was thought desirable to repeat the 1943 methods (the results of which are reported in the first three lines of Table 1), in which the eggs were scattered and bunched from fertilization and removed to separate dishes 1 hour later, in order to make sure that differences between eggs isolated at the time of fertilization and those isolated 1 hour later did not arise from some unrecognized change in technique of treating the eggs between the two years. When the 1943 methods were repeated, the results were the same as before. They are reported in the last line of Table 1. Bunches of 5–19 were found to start cleavage significantly ahead of scattered eggs. This indicates that differences in results when eggs were bunched and scattered at different times are related to the time at which the eggs were placed under experimental conditions and not to changes in treatment of eggs between the two years.

Preliminary experiments were carried out in which the rate of cleavage was measured in the usual, uncontaminated pond water, as compared with the rate in conditioned water, that is, water in which frog eggs had developed to blastula or to tail-bud stages. The conditioning eggs were kept in loosely covered dishes with as little water as possible, in an effort to keep the CO_2 concentration from rising; and since they were in a single layer, all developed at about the same rate. The supernatant liquid was milky. Eggs placed in this water were slower to cleave than in untreated water, provided the conditioned water was used immediately after the conditioning eggs were removed. The treated water produced no effect on cleavage if it was allowed to stand exposed to the air for a day or two before being tested. The retarding agent is either unstable or volatile. Carbon dioxide is known to retard cleavage (Merwin and Allee, 1943) and may have been the causative agent here in spite of efforts to keep its concentration at a minimum.

TABLE 6

RATE OF CLEAVAGE OF EGGS IN DENSE AND SPARSE POPULATIONS

No. per Dish		No. of Expts.	No. of Eggs Used		No. of Comparisons	No. of Comparisons in Which Bunched *minus* Scattered Is in Percentage Cleaved			Rate in Dense *minus* Rate in Sparse (Av. of Comparisons)
Sparse	Dense		Sparse	Dense		Over +0.6	+0.5 to −0.5	Over −0.6	
4–27	52–97	8	310	897	12	4	1	7	− 0.4
7–42	107–133	8	437	731	6	3	0	3	+ 1.2
4–37	170–187	4	241	713	4	2	0	2	− 3.0
8–20	247–286	2	137	533	2	0	0	2	−12.0

YOLK PLUG

In experiments set up to determine whether the blastopore would close more rapidly in bunched eggs than scattered ones, the following methods were used. In most cases the eggs were cut apart 1 hour after fertilization and equal numbers placed in like volumes of water. In half of the dishes the eggs were scattered, and in half they were bunched. In some cases, contributing 13 compari-

sons to the 80 included in Table 7, both the scattered and the bunched eggs were attached to a piece of paraffin paper and the paper placed in a flask. In these cases the bunched and scattered eggs were in the same flask. The eggs were allowed to develop to the yolk-plug stage with no change of water. Measurement of the plugs showed that the bunched eggs had developed faster, on the average, than had scattered eggs (see Table 7). Size differences were transposed into time differences by the use of the graph shown in Figure 1. Two of the experiments included in Table 7 contained quite a number of comparisons. In one of these experiments the bunched eggs were slower in development, but not significantly so. The other of these experiments, as well as the total of 6 experiments when considered together, showed a significantly more rapid closure of the blastopore for the bunched eggs.

TABLE 7

RATE OF CLOSURE OF BLASTOPORE OF BUNCHED EGGS COMPARED WITH THAT OF SCATTERED EGGS WHEN THERE WAS AN EQUAL NUMBER OF EGGS PER DISH

No. of Eggs per Flask*		No. of Expts.	No. of Eggs Used in Comparisons		No. of Comparisons	Closure of Bunched *minus* Closure of Fewer (Hours' Difference in Development)		*P*-Value for Comparisons
Scattered	Bunched		Scattered	Bunched		Av. of Expts.	Av. of Comparisons	
4–44	4–56	6	608	918	80	+0.2	+0.3	0.0051

* 100-cc. or 1,000-cc. flasks; all containers were alike for any given experiment.

A part, if not all, of the indicated increased rate of development of the bunched eggs may have been caused by a heightened CO_2 concentration around the grouped eggs, for CO_2 speeds the rate of yolk-plug closure (see Table 8). A preliminary experiment had indicated that a CO_2 tension as high as 7.3 cc. per liter was stimulating; however, 2 experiments with many comparisons indicated that the range of decreasing stimulation by CO_2 comes at around 3.9 cc. per liter of CO_2. The values given for the size of the yolk plugs that developed in water aerated by air approximately freed from CO_2, as compared with those in control flasks aerated by untreated laboratory air, are not based on micrometer measurements (Table 8, line 1). The eggs were killed when the blastopore was closing, at which time they were easily graded into stages by eye and given arbitrary values. These gradings were checked and rechecked, and the results fit into a trend. The same grading method was used on a series of eggs killed at regular intervals, so that size differences could be transposed into the time differences, as shown in Table 8, line 1.

If, in stoppered flasks or covered dishes with an equal amount of water, the number of eggs was increased, there would be more CO_2 with more eggs, and the eggs would be expected to develop faster in the yolk-plug stage. This was found to be the case. In one series of 7 flasks the number of eggs was increased from 8 to 84, and the CO_2 was measured after the eggs were taken out (see Table 9). It can be seen that, with 39 or more eggs per flask and with the CO_2 1.9 cc. per liter or over, the plugs were definite-

ly smaller. The increase in the amount of CO_2 in the flasks was not in proportion to the increased number of eggs present. The reason for this is not clear; it may be related to the alkaline reserve of the eggs, or it might be a result of bacterial action. Twenty-one comparisons in 7 experiments, including those in the experiment reported in Table 9, are presented in Table 10. In these comparisons the number of eggs per dish is larger for one member of the pair. The eggs are significantly faster in development at the yolk-plug stage in the flasks containing more eggs. This tabulation includes both bunched and isolated eggs, but bunched are always compared with bunched and isolated with isolated.

In the data presented in Table 11 the scattered or bunched condition and the number of eggs per dish vary, always with the bunched eggs present in larger numbers per dish. Here both local and general increasing of the CO_2 should take place, and so the stimulation should be greater than with either condition alone. There was stimulation, but one cannot analyze the relative role of the bunching and the increase in number of eggs per volume by comparisons with former observations made on the effect of these two factors separately (Tables 7 and 10), because of the variability in numbers of eggs present per dish and the differences in the volumes of water in which the eggs were contained.

TABLE 8

EFFECT OF CO_2 ON THE RATE OF CLOSURE OF THE BLASTOPORE

No. of Expts.	Type of Dish	No. of Flasks	No. of Eggs	No. of Comparisons	*P*-Value for Comparisons	Closure at High CO_2 Concentration *minus* Closure at Low Concentration (Hours' Difference in Development)			
						CO_2 High*			
						0.7	3.0	3.9	6.5
						CO_2 Low			
						0.0	0.7	0.7	0.7
1.....	Liter flask	14	979	9	0.0108	+0.4†	.....	.....	.....
1.....	Liter flask	18	331	54	0.00014	.....	+0.4	.....	.....
1.....	100-cc flask	28	250	32	0.00137	.....	+0.9	.....	.....
				32	0.1096	.....	.....	+1.0	.....
1.....	100-cc. flask	27	263	59	0.00031	.....	.....	+0.4	.....
				64	0.1096	.....	.....	.....	+0.2

* Concentrations of CO_2 in cubic centimeters per liter.
† Eggs not measured with micrometer.

TABLE 9

INCREASE IN CO_2 CONCENTRATION AND SPEEDING-UP OF YOLK-PLUG CLOSURE AS THE NUMBER OF EGGS DEVELOPING PER CLOSED 100-CC. FLASK IS INCREASED

No. of Eggs per Flask	CO_2 in Cubic Centimeters per Liter	Area* of Plugs
0.....	0.7	.....
8.....	1.1	44
9.....	1.1	45
14.....	1.2	50
28.....	1.7	44
39.....	1.9	38
66.....	2.0	41
84.....	2.7	34

* Area is in micrometer units and is the product of the two diameters (see p. 18).

In all the comparisons between yolk plugs so far, the bunched eggs were in rafts one layer thick or had an interrupted top layer. If the eggs were completely cut off from the surface, as they were in the center of a mass of eggs, the central ones were retarded in development at the yolk-plug stage, as compared with those at the surface. For example, in one bunch of 77 eggs, 33 were on the surface of the bunch and 27 were more or less inclosed; and 14 on the bottom were probably completely surrounded. The outer eggs were 0.6 hour ahead of the inner eggs in development and approximately 12 hours ahead of the ones on the bottom. The outer eggs were also 0.2 hour ahead of eggs in a comparable flask with only 12 eggs per flask. This would be expected, since the CO_2 was higher with 77 eggs per flask than when only 12 were present. Three more cases of eggs surrounded by others are listed in Table 12.

Other rafts or bunches of eggs have been observed in order to check on these findings. When a raft of eggs two or three layers thick was left barely covered with water, the eggs in the second layer from the top were slower in developing, and the bottom ones were still slower. If eggs were observed in the neural-fold stage, a center-edge difference showed up even in a raft one layer thick. The neural folds of the center eggs were slower to close.

TABLE 10

RATE OF CLOSURE OF BLASTOPORE IN RELATION TO NUMBERS PRESENT PER GIVEN VOLUME

No. of Eggs per Dish*		No. of Expts.	No. of Eggs Used in Comparisons		No. of Comparisons	More Present *minus* Fewer (Hours' Difference in Development)		*P*-Value for Comparisons
Fewer	More		Fewer	More		Av. of Expts.	Av. of Comparisons	
1–39	5–84	7	790	962	21	+1.2	+1.7	0.0000†

* 100-cc. flasks, 1,000-cc. flasks, or jelly glasses holding 125 cc.
† *t* is 11.

TABLE 11

RATE OF CLOSURE OF BLASTOPORE OF BUNCHED EGGS WITH MORE EGGS PER FLASK COMPARED WITH THAT OF SCATTERED EGGS WITH FEWER PER FLASK

No. of Eggs per Dish*		No. of Expts.	No. of Eggs Used in Comparisons		No. of Comparisons	Bunched, More *minus* Scattered, Fewer (Hours' Difference in Development)		*P*-Value
Scattered, Fewer	Bunched, More		Scattered, Fewer	Bunched, More		Av. of Expts.	Av. of Comparisons	
2–7	15–22	4	198	314	40	+1.1	+0.6	0.00032

* Jelly glasses hold 125 cc. of water.

HATCHING

Hatching is the next stage at which a clear-cut end-point can be found. According to Cooper (1936), hatching is

under the control of a secretion. Observations on hatching in one series of flasks showed that in a bunch of 50 eggs several hatched earlier than did any eggs in groups of 5 and that in the groups of 5 some eggs hatched about 12 hours before the isolated eggs began to hatch. Despite the considerable difference in the time of hatching, no difference in the developmental stage reached by the larvae from single eggs or from bunches of 5 could be distinguished when hatched larvae from single and grouped eggs were killed at the same time and observed under a binocular microscope.

One set of flasks was set up containing scattered eggs in various densities. The eggs were allowed to develop to hatching; then all were killed and examined and the CO_2 content of the water determined. In a flask in which the CO_2 concentration reached 9.0 cc. per liter, the larvae died at the time of hatching, perhaps owing to lack of oxygen. When there were fewer per flask and the CO_2 reached 6.9 cc. per liter, the larvae lived. Their gills were very slightly less developed than those in a still sparser population, where the final CO_2 concentration measured 1.4 cc. per liter. Thus CO_2, in the long run, would not seem to effect development greatly—at least under the conditions tested where the water was not changed.

DISCUSSION

The present work on frog eggs was suggested by the observations of Allee and Evans (1937*a* and *b*) on *Arbacia* eggs. They found an optimal density for rate of cleavage and early development.

CLEAVAGE

When frog eggs were under experimental conditions from the time of fertilization, the bunched eggs were found to cleave more rapidly than scattered ones. An optimal-sized bunch is indicated in which the stimulation is greater than for either larger or smaller bunches. If eggs were not grouped or isolated until 1 hour after fertilization, there was an indication of stimulation for bunches of 20, but larger or smaller groups generally showed retardation in rate of cleavage relative to scattered eggs. The difference in the amount of time the eggs were exposed to experimental conditions in these two cases would not seem to be the cause of the different results; rather, there must be some special environmental condition or reactivity of the egg existing during the first hour after fertilization.

TABLE 12

RETARDATION OF DEVELOPMENT OF FROG EGGS IN THE YOLK-PLUG STAGE WHEN THEY ARE SURROUNDED BY OTHER EGGS IN A MASS

No. of Eggs			Outer *minus* Inner (Hours' Difference in Development)
Total	Inner	Outer	
132	29	103	+5.9
131	37	94	+4.8
53	9	44	+0.7

Peebles (1929), working on *Arbacia* eggs, observed that water in which eggs had developed depressed cleavage. Allee and Evans (1937*b*) also observed this, and work reported in the present paper suggests that the same is true for frog eggs. Peebles noted speeding of cleavage when eggs were exposed to a dialyzate from cleaving eggs. She also found extracts of *Arbacia* eggs that hastened cleavage. Allee *et al.* (1942) obtained an extract of cleaving *Arbacia* eggs that stimulated cleavage. Finkel, Allee, and Garner (1942) have discussed the possibility that copper, given off by the eggs after fertilization, may be the effective agent. No work has been done on *Arba-*

cia, in which the factor or factors causing acceleration at optimal density is shown to be limited in action to a short time following fertilization; this might hold if copper was the effective agent.

Needham (1942) reports that Ashbel and, later, others have observed that an acid is produced soon after the fertilization of echinoderm eggs. One would expect this increase in acid to depress groups, since it would release CO_2 from the carbonates in the surrounding water and since CO_2 is known to depress the rate of cleavage of *Arbacia* eggs (Smith and Clowes, 1924; Haywood and Root, 1930).

Certain differences between frog and *Arbacia* eggs need to be kept in mind, even though, at present, they are hard to evaluate in relation to rate of development. The sea-urchin egg has already gone through both maturation divisions, while the frog egg has yet to give off the second polar body after the sperm enters. The fertilization membrane rises in *Arbacia* in 4 minutes, while in the frog egg it takes three-quarters of an hour to an hour at 16° C. under experimental conditions.

When frog eggs are bunched or scattered at fertilization, a number of factors, not present in the work on *Arbacia* or in the work on frog eggs that are scattered or bunched an hour after fertilization, are introduced, differentiating between the bunched and scattered eggs. These factors must be considered as possible reasons for the differences observed between eggs isolated or bunched at fertilization and those similarly treated an hour later. Scattered eggs show a lower fertility, a state that may be related to the fact that these eggs may be more shaken about than the bunched eggs at the time the dish is shaken to get the eggs bunched and scattered. It has been pointed out earlier that eggs are very sensitive to mechanical disturbance after fertilization. Disturbance prevents cleavage and may slow it, but this point has not been settled in the present work. There might be a differential rate of penetration of the bunched and scattered eggs by the sperm; the scattered eggs are more thoroughly washed; also, they are faster to turn.

Another possible difference between isolated and bunched eggs during this period deserves brief attention. According to Needham (1931), Bialaszewicz and Bledowski report that freshly ovulated frog eggs give off a considerable amount of CO_2. Needham (1942) also reports that Dalcq and Pastells and Brachet find that much of the CO_2 is discharged in the oviduct and that, only when the CO_2 is low enough, will fertilization take place. The CO_2 might become lower sooner in the isolated eggs and fertilization take place sooner; then they should cleave sooner. The CO_2 given off by the grouped eggs would raise the CO_2 tension around them and slow the appearance of the first furrow, since any increase in CO_2 has this effect (Merwin and Allee, 1943). In both these cases the expected results would be a slowing of the bunched eggs relative to the scattered eggs, and this is contrary to the evidence reported in this paper. Any increase in CO_2 has been shown to delay the appearance of the first few cleavage furrows for *Arbacia* eggs (Smith and Clowes, 1924, and Haywood and Root, 1930); it has the same effect on the cleavage of frog's eggs. In spite of this, denser populations of *Arbacia* eggs and groups of frog eggs cleave more rapidly than sparse populations or scattered eggs.

Osmotic conditions around scattered eggs may differ from those around bunched ones during the first hour. Needham (1931) reports the work of several investigators that indicates that

after fertilization frog eggs give off salts into the perivitelline space. Although no reports have been found in the literature regarding the diffusion of substances out into the surrounding water from the perivetelline space during the first hour after fertilization, McClendon (1915) showed that salts diffuse out at a much faster rate during the first 7 hours after fertilization than later. Voss (1926) found that the perivitilline space of embryos in the medullary fold stage (the youngest stage he observed) were smaller in crowded eggs than in less crowded ones.

Allee *et al.* (1942) do not think that the heat of metabolism is an important factor in accelerating the rate of cleavage in denser populations of *Arbacia* eggs, and no proof that it is important in causing bunches of frog eggs to cleave more rapidly than scattered ones could be obtained in the present experiments. The observed differences in rates of cleavage for frog eggs, isolated or bunched at fertilization, would require approximately 0.06° C. difference in temperature, based on the effect of temperature on the development of *R. pipiens* as determined by Atlas (1935). Observations with two Beckman thermometers, calibrated to each other and placed under various conditions, indicated that a range of 0.03° C. between dishes might exist, but not differentially for experimental and control dishes. When one thermometer was placed among eggs or was coated with eggs, it did not reveal any consistant change in temperature when compared with the other thermometer.

YOLK PLUG

By the time the yolk-plug stage is reached, the reaction to CO_2 has changed; and, while increased CO_2 tension depresses cleavage, it accelerates blastopore closure. It has other processes to work on besides cleavage—for instance, migration of cells. Approximately 3.0 cc. per liter of CO_2 caused most rapid development at this stage. Later, in the adult body, the cells operate at a much higher CO_2 concentration.

Bellamy (1922) observed stimulation of the development of *R. pipiens* in the yolk plug and also in the larval stages in a N/5,000 and N/20,000 HCl solution in well water. He suggested that CO_2 released from the carbonates by the acid was the stimulating substance. In his experiments the water was changed every day, while this was not done in the present experiments. This may explain the fact that Bellamy obtained stimulation lasting to hatching, while this stimulation did not continue in the present experiments to any measurable extent. Vernon (1895), working with echinoderm larvae, observed a slight trend toward stimulation by CO_2; and he found that concentrations up to the critical one did not interfere with development to any extent.

HATCHING

It has been pointed out that bunched eggs hatch sooner than isolated ones. Cooper (1936) obtained a hatching secretion by removing the membranes from eggs and allowing the eggs to develop to the stage at which they would normally hatch. The enzyme obtained in this manner may be concentrated enough to dissolve the jelly from other eggs in 1 hour. Bliss (1940) found that 10 per cent trypsin would produce the same effect and did not affect the rate of development but that development stopped in the presence of tryptic digest products. Shumway (1940) observed that embryos in his Stage 19 hatched if they were shaken, while those in his Stage 20 hatched spontaneously. In the present work, groups of 5 eggs hatched sooner than isolated ones, and observa-

tions of the larvae killed after all had hatched revealed no difference in development. If groups were larger, the center ones were retarded in development; and these did hatch sooner, relative to their growth stage—at least for Shumway's Stage 19 or earlier; but accurate staging was not carried out. The earlier hatching, relative to the development of the larvae, may be due to the earlier stimulation of the larvae to move and rupture the membranes or to a more effective action of the enzyme, owing to a higher concentration, caused by crowding, or to more favorable chemical conditions around crowded eggs.

The present study extends our knowledge about optimal populations to the early development of another species—this time a vertebrate. The wider aspects of these and related phenomena are discussed by Allee (1938). The rate of development is a favorable indicator for use in such population studies and may well have ecological significance, as will be discussed later. Observed modifications to date are relatively small in most cases and consist of slight variations in rate of cleavage or in rate of later development. These are often, but not always, temporary and subject to later compensation.

The egg cell is not dependent on other cells for growth-stimulating substances, the need of which in the case of body cells shows up in tissue culture. Bacterial cells are often dependent on each other in that a certain size inoculum is necessary for survival and growth. Garrod (1936) suggests, in explanation, that certain bacterial cells produce a substance necessary to each other and must have it in a certain concentration. Eggs shed into the water are independent of other organisms except sperm of the same species. *Arbacia* eggs are known to secrete substances relative to fertilization, such as fertilizin (Lillie, 1919); but these are probably not significant in the present study and can be excluded with certainty for *Arbacia* eggs and for frog eggs isolated an hour after cleavage. Other substances known to be produced during the early development of the *Arbacia* eggs are copper and an acid; these have been discussed. Other metabolic products, such as CO_2, ammonia, and urea (Brachet, 1939), or special secretions, such as hatching enzymes, may become concentrated around grouped eggs and modify the rate of development or the time of hatching.

If group-initiated modifications were steadily advantageous generation after generation, one would expect selection to have resulted in eggs being laid in masses of some size. The fact that some species of animals—frogs among them—lay their eggs in bunches may be an indication of such advantages. The benefit derived from bunching that is most frequently mentioned in the literature is that the large mass of jelly holds heat absorbed from the sun's rays by the darkly pigmented embryos and so keeps the temperature of the mass above that of the surrounding water. Moore (1939) notes that clumps of eggs are characteristic of early spring breeders—at least in the temperate zone. The significance of this is not clear, although a possible temperature effect may be indicated.

The indication from the work reported in this paper of a sensitive period following fertilization suggests that bunched eggs might be more protected from mechanical injury during that time. This possible mechanical protection of eggs in bunches suggests certain comparisons with the advantages of a large graft in transplanting tissues when mechanical or sudden chemical changes may harm small grafts more than large ones (Waterman, 1936). Developmental

acceleration of grouped eggs by CO_2, such as has been reported in the present paper for the yolk-plug stage, would not seem to be important under ordinary circumstances. Significant speeding of total development or of hatching could be of importance to frogs when the eggs are laid in ponds which dry up or in which the water level may fall.

Other types of biotic relations, including predation, may also have affected the evolution of the laying of eggs in groups. The survival values involved are complex and call for experimental analysis rather than a priori speculation.

Whatever the larger implications, certain definite results have been obtained as a result of the present study. These will be summarized in the following paragraphs.

SUMMARY AND CONCLUSIONS

Frog eggs (*R. pipiens*) that were bunched at the time of fertilization cleaved more rapidly than those that were scattered. Bunches ranging in size from 7 to 20 eggs were consistently significantly ahead of scattered eggs; those of from 21 to 60 eggs in size were not significantly ahead, although a trend may have been present. There are indications that bunches smaller than 7 eggs were less accelerated than those of 7–20 eggs. Thus, an optimal-sized bunch accelerates the first few cleavages. The percentage of fertility of the scattered eggs was lower than that of the bunched eggs.

If the eggs were not placed in groups or scattered until an hour after fertilization (after the eggs turned), there was a slight, almost significant, tendency for the groups of 20 eggs to cleave faster; but both smaller and larger groups cleaved slower than accompanying scattered eggs; the groups of 40 eggs, significantly so. The depressing effect of bunching under these conditions may be the result of an increased CO_2 concentration. The factor or factors causing acceleration are not known.

Closure of the blastopore took place sooner in eggs in a group one layer thick than in scattered eggs, and it was faster if there were more eggs per volume of water. This speeding of development was probably the result of an increased CO_2 concentration. A content of approximately 3 cc. of CO_2 per liter was more effective than higher or lower concentrations in speeding the closure of the blastopore.

If eggs were in the center of a mass, they were retarded in the yolk-plug stages and later. Such eggs have not been studied in the cleavage stages. The central eggs in a group one layer thick were noticeably retarded in the neural-fold stage and later.

Grouped eggs hatched sooner than isolated ones, provided the groups were not so large that development was retarded for the centrally placed embryos.

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EBONY: A GENE AFFECTING THE BODY COLOR AND FECUNDITY OF TRIBOLIUM CONFUSUM DUVAL[1]

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A MUTATION, in order to have value for experimental population studies, must display at least these three properties: first, the phenotypic character should be quite visible and thus capable of rapid diagnosis; second, the mutant gene should not have too deleterious an influence on the organism's reproduction and viability, although a moderate effect of this type is desirable; and, third, it must be possible to husband the mutant forms successfully. One such mutant, which satisfies these requirements for the red-rust flour beetle, *Tribolium castaneum*, has already been described (Park, 1937*a*); and its distribution in laboratory populations under various environmental conditions is being analyzed by Kollros in this laboratory. This mutation was named "pearl" and is inherited as a simple, Mendelian, autosomal recessive (pp). Pearl-eye beetles have a marginal ring of black facets surrounding the eye. The remaining or central facets seem to be colorless in contrast to the normally appearing eye for this genus, which is uniformly black.

A new body color of *T. confusum* recently appeared in several experimental vials in which census counts were under way.[2] This form is essentially black to the eye and is readily distinguished from the red-brown color of the normal type. We immediately suspected that the darkening had a genetic basis and planned a series of matings to test the point. It is shown in this paper that the effect is due to a single, recessive, autosomal gene inherited according to Mendelian expectations. This gene, named by us "ebony," for obvious reasons, is symbolized in the homozygous state as "ee"; the normal homozygote is thus "EE"; and the heterozygote, appearing phenotypically normal, is "Ee." Since the student of populations is interested in the statistical consequences of reproduction and development and since we are using this mutant in further experimentation, the effect of the gene on fecundity, fertility, and rate of larval and pupal development is also analyzed. In addition, we present data on the degree or strength of pigmentation in normal and ebony *T. confusum* and normal *T. castaneum*.

Enough genetic work has been done on Coleoptera to warrant perhaps a brief review of certain of the more significant findings. The remarks that follow are neither critical nor comprehensive. They are included only to indicate those species about which genetic knowledge is available and to lead the reader further into the literature if he so wishes. We are not concerned here with studies that describe "varieties" of beetles or instances of variability in pattern for which the genetic mechanisms are not known.

Coccinellidae.—The ladybird beetles have probably been more extensively

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[2] The dark forms were first observed by Mrs. Arthur H. Davis, a member of our laboratory group.

studied from the genetic viewpoint than any other family. Emphasis has been placed on the color and spotting pattern of the elytra as the phenotypic characters. A recent paper by Shull (1943) identifies a gene for *Hippodamia sinuata* and reviews extensively the literature dealing with this group. Shull finds that absence of spots ("spotless") on the elytra differs from the presence of spots ("spotted") by a single gene. Spotless "is not quite dominant; though a few of the heterozygotes are strictly spotless, most of them have reduced spotting. There is little or no overlapping of the phenotype of the heterozygote and that of the spotted homozygote" (p. 336). Shull identifies the spotless gene as "S"; its allele for spotted as "s."

In *Coelophora inaequalis*, Dobzhansky (1933) analyzes the published data of Timberlake (1922) and concludes that a series of multiple alleles are present that affect spotting and color. He finds that a "nine-spotted" condition is dominant to "normal" and that the latter is dominant to "black." In *Harmonia axyridis*, eight patterns are now recognized in a multiple allelic series. Dominance between these alleles is "sometimes complete, sometimes partial or lacking" (Shull, 1943, p. 330). These findings are based on the work of Tan and Li (1932–33) and of Hosino (1940).

Adalia bipunctata has been studied by Lus (1928), who concludes that eight different forms are dependent on genes in the same locus. Timoféeff-Ressovsky (1940) is not convinced that the evidence for this conclusion is entirely justified, however. In the same paper Lus also reports on three alleles that affect the elytra of another species of *Adalia*, *A. decempunctata*.

It seems fair to conclude that elytra color and pattern in ladybird beetles is definitely influenced by genetic factors and that multiple alleles have been demonstrated in several instances.

There are also a series of studies on coccinellids describing phenotypic variability from sampled natural populations but which do not analyze the hereditary mechanisms involved. Also, several workers, notably Dobzhansky (1924) and Tan and Li (1932–33), have studied the statistical proportions of various types of *H. axyridis* in different natural populations—particularly in relation to geographic range and climate. These reports are ably reviewed by Shull (1943).

Chrysomelidae.—McCracken (1907) worked with the beetle *Melasoma scripta*. This species is typically found in natural populations with two phenotypes predominating: spotted-brown elytra and black elytra. McCracken noted that occasionally a sport appears which is totally black—i.e., with black head, thorax, and elytra. The thorax in the two usual color forms is black in the center, widely emarginated with brick red. On the basis of experimental breeding she identifies the gene "S," which is apparently responsible for the spotted-brown phenotype; the gene "B," for the black elytra; and the combination "AB," for the totally black forms. She concludes that the order of dominance is S>B>AB. It is not entirely certain that all these relationships are completely understood, since McCracken observes that "there is no general adherence to Mendelian proportions in the behavior in inheritance of the sport AB in Melasoma scripta but it behaves as a Mendelian recessive in first crosses with both S and B, breeding true from hybrid S" (p. 237). In any event, it seems safe to conclude that genes for color and pattern are identified and that dominance relations are established.

Tenebrionidae:(*a*) *Tenebrio molitor.*—An impressive amount of genetic work

has been carried on by several investigators with the meal worm *Tenebrio molitor*. The key references to these studies are: Arendsen Hein (1920, 1924), Ferwerda (1928), and Schuurman (1937). It is outside the scope of this paper to review these findings in detail. It is appropriate, however, to indicate the characters studied and something of the state of knowledge concerning their genetic basis. Arendsen Hein, who really initiated this work on a large scale, identified a number of genes affecting larval body color, certain appendage abnormalities, head-groove deviations, and eye color. Upon his death the work was continued by Ferwerda and then later extended, especially in physiological directions, by Schuurman. In brief, these are the more significant findings:

1. Eye Color: Four colors are described and named as follows: black, red, yellow, and flesh colored. These colors are based on a series of interacting genes. The following genotypes are known for female beetles:

FFGGHH	black eye
ffGGHH	red eye
FFGGhh	yellow eye
ffGGhh	yellow eye
FFggHH	flesh-colored eye
ffggHH	
FFgghh	
ffgghh	

2. Appendage modifications: Four types are described and named, as follows: normal type, type with abnormal tarsus, type with flattened (*Abgeplatteter*) antennae, and reduced type. They are explained on a system of three interacting genes, and the following genotypes are known:

CCDDEE	normal
ccDDEE	abnormal tarsus
CCddEE	flattened antennae
CCDDee	reduced type

3. Larval color types: Three colors of the larvae are recognized; and these are, in order of dominance: orange > umber brown > yellow brown. Ferwerda considers these colors due to a triple allelic series and identifies the three types as AA, a_2a_2, and a_1a_1, respectively.

4. Head groove: A gene has been identified that causes the head groove to be V-shaped rather than normal in configuration. This gene, B, is dominant over the normal groove, b.

5. In certain of the above relationships, illustrations of linkage, lethal factors, and sex-linked inheritance have been unearthed. Also, "eye mosaics," in which the eye is part red and part black (*Gefleckt*), have been studied and a genetic hypothesis advanced in explanation.

b) *Tribolium.*—There are only two genes identified for this tenebrionid genus. One is the "pearl" eye already mentioned, which occurs in *T. castaneum;* the other, the gene "ebony" appearing in *Tribolium confusum* and described in this paper. We frequently see and study in the laboratory various sorts of modifications, especially of the appendages; but to date these have proved to be, on exploration, only individual, somatic variants and not hereditary in character. The chances are, as Roth (1944) has shown, that many of these variants are caused by a substance secreted by special glands, which interferes with normal development. This substance has recently been identified chemically by Alexander and Barton (1943) as "ethylquinone." Park and Davis (1944) have shown that the fecundity of *T. castaneum* can be increased about 6 per cent above that of stock-culture beetles through selection. This is patently a genetic matter, but no actual gene or genes have been identified.

Curculionidae.—Breitenbecher (1921) has described mutations for the four-spotted cowpea weevil, *Bruchus quad-*

rimaculatus, which involve elytra and body color and which seem to be included in a single, multiple, allelic system. The findings are well summarized as follows:

> . . . . The order of dominance for these four-color factors was red, black, white, and tan (the wild type). The females and males of the wild type have tan elytra and bodies, while the females of the white mutant cultures have white elytra and bodies and the males have tan elytra but gray bodies. The black mutant stock shows a more pronounced dimorphism, since its females had black elytra and bodies, and its males tan elytra but grayish-black bodies. A lesser difference is seen in the red mutation, for its females had red elytra and bodies, while its males had tan elytra and reddish-gray bodies. It is evident from this description that every male had tan elytra. In conformity with an allelomorph series, the following formulae were designated by the author: red (RR), black (R^bR^b), white (R^wR^w), and tan or wild (rr). These formulae also indicate the order of dominance [Breitenbecher, 1922, pp. 10–11].

In a later paper Breitenbecher (1922) points out that *Bruchus* occasionally displays "elytral mosaics." This is a situation in which, in addition to normal bilateral colors such as red left wing and red right wing, beetles appear which are red-black, black-red, black-tan, tan-black, and white-tan. A hypothesis is advanced to explain these mosaics.

THE TEST CROSSES

In all the test crosses reported in this paper a single pair of *T. confusum*, not over 3 weeks old as imagoes and isolated as pupae so as to be virgin, is introduced into a vial (9.5 × 2.3 cm.) containing 8 gm. of standard laboratory medium. This medium consists of fine-sifted whole-wheat flour, 95 per cent by weight, and dry brewers' yeast, 5 per cent by weight. Both flour and yeast are screened through a No. 5 silk bolting-cloth sieve to collect particles of the desired size and then thoroughly mixed together for 2 hours in a mechanical rotating device.

The normal beetles (EE) were taken from laboratory stocks in which no ebony forms had ever appeared. The ebony beetles (ee) were collected from one of the several experimental vials in which they were first noted and then were self-mated for two generations to provide the mutant stock needed for the mating experiments.

After a vial has been set up and after larvae are present, the parental imagoes are removed before the next-generation adults appear. The original parents are immediately transferred into duplicate vials containing fresh medium. This procedure essentially doubles the size of the counts for each respective parental pair. All data reported are based on the summation of progeny from the original vial plus the duplicate vial. Each culture is usually examined three times, extending over a 2–3-week period. At each examination the imagoes are counted, sexed, and classified by phenotype; and these data are recorded. If a new seeding is needed from a respective vial for a subsequent mating, pupae are isolated and utilized for that purpose.

The cultures are kept in a dark incubator at a mean temperature of 29° C. (maximum variation: ±1°) and at a relative humidity of 60–75 per cent. In so far as possible, all handling and examination procedures are standardized.

The data summarizing the above matings are presented in Table 1. An examination of this table supports the hypothesis that ebony is a single, recessive, autosomal gene. The major points in evidence of this are as follows:

1. The F_1 generation (Ee) is phenotypically normal in respect of body color (matings I and II).

2. The F_2 generation segregates into the expected 75 per cent normal (both EE and Ee) and the 25 per cent ebony (ee) (mating IV).

3. The two backcrosses to the homozygous recessive (ee) yield the expected ratio of 50 per cent normal (Ee) to 50 per cent ebony (ee) (matings V, VI, VII, and VIII).

4. The two backcrosses to the homozygous dominant (EE) yield only progeny that are phenotypically normal; unquestionably, one-half of these are EE, the other half Ee (matings IX, X, XI, and XII).

5. Ebony mated with ebony yields only ebony (mating III and the self-mated ebony stocks).

Certain discrepancies in the ratios reported in Table 1 merit attention even though these do not modify the basic genetic conclusion about the gene ebony and even though they cannot be precisely explained. Every phenotypic and sex ratio was tested statistically against an expectation of either $\frac{1}{2}:\frac{1}{2}$ (for sex ratios and recessive backcrosses) or $\frac{3}{4}:\frac{1}{4}$ (for the F_2 progeny).[3]

TABLE 1

SUMMARY OF THE TEST CROSSES

Genetic Mating		No. of Vials	No. of Normal Progeny		No. of Ebony Progeny		Percentage of Total Progeny That Are—	
No.	Type		Males	Females	Males	Females	Normal	Ebony
I	EE♂♂ × ee♀♀	10	1,086	1,108	0	0	100.0	0
II	ee♂♂ × EE♀♀	10	1,432	1,523	0	0	100.0	0
I and II	EE × ee	20	2,518	2,631	0	0	100.0	0
III	ee × ee	6	0	0	502	534	0	100.0
IV	Ee × Ee:							
	Parents from No. I	5	451	563	171	174	77.9	22.1
	Parents from No. II	5	328	380	87	114	74.6	25.4
	Both	10	779	943	258	288	75.9	24.1
V	Ee♂♂ × ee♀♀	5	223	289	218	237	52.9	47.1
VI	ee♂♂ × Ee♀♀	5	254	319	259	314	50.0	50.0
V and VI	Ee × ee	10	477	608	477	551	51.3	48.7
VII	Ee♂♂ × ee♀♀	5	325	349	283	316	52.9	47.1
VIII	ee♂♂ × Ee♀♀	10	681	709	585	613	53.7	46.3
VII and VIII	Ee × ee	15	1,006	1,058	868	929	53.4	46.6
IX	Ee♂♂ × EE♀♀	5	480	548	0	0	100.0	0
X	EE♂♂ × Ee♀♀	3	292	357	0	0	100.0	0
IX and X	Ee × EE	8	772	905	0	0	100.0	0
XI	Ee♂♂ × EE♀♀	5	508	590	0	0	100.0	0
XII	EE♂♂ × Ee♀♀	5	537	474	0	0	100.0	0
XI and XII	Ee × EE	10	1,045	1,064	0	0	100.0	0

This test, applied to matings IV, V,

[3] This was done by the formula

$$\frac{\text{Observed deviation from expected}}{\text{Its probable error}},$$

where P.E. $= 0.6745\sqrt{npq}$, or, in the case of percentages, used in certain instances, where P.E. per cent $= 0.6745\sqrt{pq\left(\frac{1}{N_1}+\frac{1}{N_2}\right)}$.

and VI, shows that the deviations from the expected are well within the limits of random sampling. The test applied to the second, recessive backcross matings (VII and VIII), however, shows that significantly more normal beetles (Ee), and significantly less ebony, are counted as imagoes than anticipated on the assumption of $\frac{1}{2}:\frac{1}{2}$. In other words, the actual percentages, 52.9–47.1 and 53.7–46.3, show an excess in favor of normal type. This excess, although statistically significant, is not detrimental to the hypothesis that ebony is a single recessive gene. It does suggest that the normal type has a slight selective advantage over the mutant. This matter can be explored further in population experiments.

Analysis of the sex ratios brings out the point that male imagoes do not appear in the counts with as high frequency as females. If every sex-ratio entry in Table 1 is examined, it is noted that there are 27 instances in which the female count exceeds the male and only 1 instance in which the reverse is true. Obviously, not all these deviations are significant, but a number of them are. Of 31 sex-ratio comparisons, 13 are cases for which the sex ratios do not differ significantly from the expected, and 18 are cases for which the difference does exceed three times its probable error. The distribution of males and females by phenotypes for the total experience is as shown in the accompanying table. These three differences in sex ratio all exceed their respective probable errors by four times. Also, sex ratio is unaffected by phenotype.

It is thus incontestible that, so far as these experiments go, there is some sort of influence operating in favor of the females. The nature of this is not apparent. Whether the difference is one obtaining at the time of oviposition, i.e., a zygotic difference, or whether it is due to some selection operating within the cultures is not clear. It seems a priori that the latter is a more likely hypothesis.

	Number	Per Cent
All normal males	6,597	47.78
All normal females	7,209	52.22
Total normal beetles	13,806	100.00
All ebony males	2,105	47.76
All ebony females	3,302	52.24
Total ebony beetles	5,407	100.00
Total males	8,702	47.78
Total females	9,511	52.22
Grand total	18,213	100.00

FECUNDITY RATES

Experiments were conducted on a rather large scale to see if the ebony gene has any significant influence on egg production in addition to its demonstrated effect on pigmentation. There would be nothing surprising in either this demonstration, that a single gene frequently affects several characters both morphological and physiological, or the reverse, that a single character can be affected by several genes.

The fecundity assays are carried out according to a procedure standardized in this laboratory (see Park and Davis, 1944). One pair of imagoes, 2–3 weeks old, is introduced into a shell vial containing 4 gm. of the flour-yeast medium. This vial, along with its counterparts, is lightly stoppered with a cotton plug and placed in the incubator under the same conditions of temperature and humidity described earlier. After 5 days the contents of each vial are emptied into a sieve, the imagoes gently removed with spatula and brush, and the medium passed through a No. 5 bolting cloth. The eggs retained in the sieve

are counted with a hand tally, and the number recorded. The beetles are returned to their respective vials, to which has been added 4 gm. of fresh medium (the old medium being discarded), and then put back into the incubator for 5 additional days. It has been shown that "conditioning" of the flour by the population is an important factor in reducing fecundity (Park and Woollcott, 1937; Crombie, 1943). This is essentially eliminated by changing the medium each 5 days.

tions. The data are summarized for each count in Table 2, in which is tabulated the mean number of eggs and its probable error; the standard deviation of the distribution; the coefficient of variability; and the number of pairs or vials (n) entering into the computations. The counts are also grouped in the table to include the first 30-day period, the second 30-day period, and the total or 60-day period. The mean fecundity trends are graphed in Figure 1.

An examination of Tables 2 and 3

TABLE 2

BIOMETRIC CONSTANTS FOR THE FECUNDITY OF HOMOZYGOUS-DOMINANT, HOMOZYGOUS-RECESSIVE, AND HETEROZYGOUS *T. confusum*

(Eggs per Pair per 5-Day Period)

Days	EE×EE				Ee×Ee				ee×ee			
	Mean (Eggs)	Standard Deviation (Eggs)	Coefficient of Variability (Per Cent)	n (Pairs)	Mean (Eggs)	Standard Deviation (Eggs)	Coefficient of Variability (Per Cent)	n (Pairs)	Mean (Eggs)	Standard Deviation (Eggs)	Coefficient of Variability (Per Cent)	n (Pairs)
0–5....	48.3±1.83	18.6	38.5	47	54.4±1.77	18.4	33.8	49	48.1±1.40	14.5	30.1	49
5–10....	52.7±1.61	16.4	31.1	47	57.1±1.73	17.8	31.2	48	51.4±1.37	14.1	27.2	48
10–15....	55.9±1.42	14.4	25.8	47	54.1±1.46	14.7	27.2	46	51.2±1.14	11.7	22,8	48
15–20....	54.2±1.50	15.3	28.2	47	55.7±1.57	15.8	28.4	46	47.9±1.21	12.4	25.9	48
20–25....	56.9±1.66	16.9	29.7	47	59.0±1.33	13.2	22.4	45	51.9±1.17	11.9	22.9	47
25–30....	54.1±1.59	16.2	29.9	47	56.0±1.64	16.5	29.5	46	48.4±1.53	15.6	32.2	47
0–30....	53.8±0.65	16.2	30.1	282	56.0±0.66	16.4	29.3	280	49.8±0.54	13.6	27.3	287
30–35....	55.9±1.74	17.5	31.3	46	58.9±1.56	15.7	26.6	46	48.0±1.15	11.7	24.4	47
35–40....	53.1±1.53	15.2	28.6	45	54.9±1.61	16.2	29.5	46	44.1±1.22	12.4	28.2	47
40–45....	50.7±1.55	15.6	30.8	46	57.1±1.31	13.2	23.1	46	44.2±1.27	12.9	29.2	47
45–50....	46.8±1.41	14.0	29.9	45	45.0±1.25	12.6	28.0	46	35.2±1.47	14.9	42.3	47
50–55....	43.3±1.29	12.8	29.6	45	40.7±1.39	14.0	34.4	46	34.4±1.30	13.1	38.1	46
55–60....	37.0±1.25	12.4	33.5	45	34.6±1.56	15.7	45.4	46	29.5±1.08	10.8	36.6	45
30–60....	47.8±0.66	16.1	33.7	272	48.5±0.70	17.3	35.7	276	39.1±0.60	14.9	38.1	279
0–60....	50.8±0.47	16.5	32.5	554	52.3±0.49	17.2	32.9	556	44.6±0.42	14.9	33.4	566

The fecundity experiments were started with 50 vials each containing a pair of EE beetles; 50 containing a pair of Ee beetles; and 50 containing ee. If a pair proves to be sterile, it is removed from the study and is not represented in the final data summary. In several instances when a pair became completely sterile midway in the course of the experiment its place was filled with a comparable replacement vial. The assay runs for 60 days or for twelve examina-

(the latter, statistical analysis) leads to the following conclusions:

1. Ebony beetles have a fecundity rate lower by approximately 10 per cent than do either homozygous or heterozygous normal beetles. This difference is highly significant statistically and shows that the ebony gene, when homozygous, has a moderate but real effect on oviposition as well as on pigmentation.

2. There is no significant difference in

fecundity between EE and Ee beetles. The latter tend to oviposit a few more eggs, but the fact has no statistical validity.

3. There is no consistent difference between the coefficients of variability of the three mating types. The relative variation about the mean is essentially similar, irrespective of the genotype of the parents.

4. All three series show the "aging effect" characteristic of *Tribolium*. Maximum reproduction is attained during the first 4–6 weeks after initial mating.

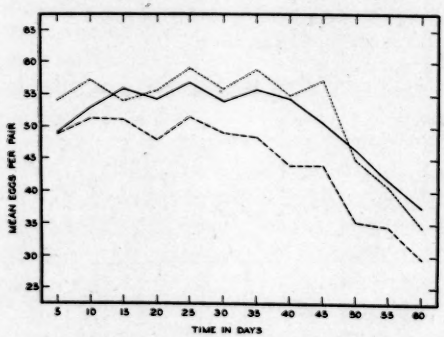

FIG. 1.—Mean fecundity in relation to age. Solid line, EE beetles; short dotted line, Ee beetles; long dotted line, ee beetles.

Fecundity is significantly greater by about 11–21 per cent for the 0–30-day period than for the 30–60-day period. This is particularly well shown by the downward slope of the three curves in Figure 1. The effect has also been recently reported for *T. castaneum* by Park and Davis (1944).

Before leaving the question of fecundity it is instructive perhaps to point out that the oviposition rates described in this paper are considerably lower than those reported in another connection by Park and Davis (1944), using the same stocks and the same experimental manipulations. This is brought out in Table 4 by comparing the means listed in Table 2 with those presented in the other study.

This significant difference in fecundity between one block of observations and another run at a different date is a typical finding, so far as our experience goes. It is true despite the fact that environmental and genetic factors have been controlled with considerable care. The difference, of course, indicates that uncontrolled factors are still present and makes it mandatory that each experiment have its own control rather than relying on one run earlier. The general point is well recognized by *Drosophila* workers: the environment for that form is much harder to maintain constant in the laboratory than for *Tribolium*. It has also been the subject of analysis in papers by Wright (1922 *a*, *b*), dealing, among other things, with fertility of guinea pigs, and by Wright and Eaton (1929), dealing with weights of guinea pigs.

FERTILITY RATES

An impressive amount of data accumulated for *T. confusum* indicates that, while fecundity is readily influenced by such diverse ecological factors as temperature, humidity, quality, and quantity of medium, flour conditioning, aging of parents, population density, frequency of copulation, and so on, fertility, or egg hatchability, is remarkably resistant to external stimuli.[4] In short, an egg, once laid, has just about as likely a chance of hatching if it comes from an imago pair living under optimal condi-

[4] Key references to papers describing these phenomena are: For fecundity: Chapman and Baird (1934), temperature; Holdaway (1932), humidity; Park and Burrows (1942), medium; Park and Woollcott (1937), conditioning; Park and Davis (1944), aging of parents; Park (1933) and Crombie (1943), copulation frequency and population density. For fertility: Park and Woollcott (1937). General summarizing references are: Ford (1937) and Park (1941).

tions as it does if the imago pair lives under suboptimal conditions.

In the present experiments the possible effect of the ebony gene on fertility is studied. Two hundred and fifty eggs were collected at random from cultures of homozygous-normal and 250 from homozygous-recessive beetles. These are then placed in clean Petri dishes and examined at frequent intervals for first-instar larvae. As the larvae hatch, the fact is noted; and the empty egg case is removed from the dish and discarded. This experiment provides a simple measure of fertility of the two genotypes. The data are summarized as shown in the accompanying table. There is clearly no significant difference between the fertility of eggs from EE parents and those from ee parents. The probability that this difference departs from an expectation of $\frac{1}{2}:\frac{1}{2}$ exceeds 0.5 (0.9 times its probable error). Thus, fertility again emerges as a relatively fixed characteristic when contrasted with fecundity, but this time in the presence of a possible genetic, rather than an ecological, differential.

Genotype	No. of Eggs Observed	No. of Resulting Larvae	Percentage of Fertility
EE	250	210	84.0
ee	250	198	79.5

TABLE 3

STATISTICAL COMPARISONS OF THE MEAN FECUNDITY RATES LISTED IN TABLE 2

The Comparison	Mean Difference	Difference in Favor of—	Difference / P.E. Difference	Probability
For 0–60-day totals:				
EE with ee	6.18	EE	9.96	<0.0000
EE with Ee	1.48	Ee	2.18	0.1378
Ee with ee	7.66	Ee	11.87	<0.0000
For 0–30-day totals:				
EE with ee	4.0	EE	4.8	0.0015
EE with Ee	2.2	Ee	2.4	0.1055
Ee with ee	6.2	Ee	7.4	<0.0000
For 30–60-day totals:				
EE with ee	8.7	EE	9.8	<0.0000
EE with Ee	0.7	Ee	0.7	>0.5
Ee with ee	9.4	Ee	10.2	<0.0000
For 0–30- and 30–60-day totals within matings:				
EE	6.0	0–30	6.5	<0.0000
Ee	10.7	0–30	13.7	<0.0000
ee	7.5	0–30	7.8	<0.0000

TABLE 4

FECUNDITY COMPARISONS OF RATES REPORTED IN THIS STUDY WITH THOSE REPORTED BY PARK AND DAVIS (1944)

Interval (Days)	Mean Fecundity per Pair per 5 Days	
	In this Study	In Park and Davis (1944)
0–30	53.8±0.65	64.2±0.73
30–60	47.8±0.66	56.8±0.68
0–60	50.8±0.47	60.4±0.53

RATE OF METAMORPHOSIS

The last factor of population significance to be analyzed is the rate of metamorphosis of EE and ee *T. confusum*. This rate is defined as the time required for the forms to pass through their larval and pupal periods. The larval period extends from hatching of the first-instar larva to pupation; the pupal period, from pupation to eclosion.

Eight grams of flour-yeast medium are introduced into the shell vials, after which each vial receives a single first-instar larva. The larvae are collected as follows: several hundred eggs of the desired genotypes are placed in clean Petri dishes and watched at frequent intervals; and, as the larvae hatch, they are removed with a small brush and set aside. In this way enough larvae can be collected to start the experiment and, at the same time, assure that there is little age difference between them when they are introduced into the vials. The maximum age allowed is 6 hours, and most larvae were not over 3 hours. The vials are kept in the incubator under the same conditions of temperature and humidity that obtained for the test crosses and the fecundity and fertility experiments. Thirty-five EE vials and 35 ee vials were started. Considerable larval mortality in both series reduced these numbers to 23 and 28 vials, respectively. Further pupal mortality reduced the numbers to 22 and 24, respectively.

The vials are not disturbed during most of the larval period. As pupation, and, later, eclosion, approaches, the vials are examined often enough to permit timing of the two periods with a maximum error of 6 hours. This is further refined by averaging the data. When the pupae appear, they are sexed by microscopic examination of the external genitalia (Park, 1937*b*). This makes it possible to divide the vials into those that contained male larvae (as well as pupae) and those that contained females and thus to assay the possible relation of sex to rate of postembryonic development.

The findings are summarized in Table 5, in which the tabulation of the data is self-evident. The major point that emerges when the table is studied can be simply put: there is no effect of the ebony gene, or of sex, on either larval or pupal duration. When statistical comparisons are made between EE and ee larval and pupal rates and between male and female rates for larvae of the same genotype, no significant difference in relation to its probable error is found. This is the expected result for pupal-stage duration, since experience has shown that this period, like fertility, is relatively fixed and difficult to shorten or extend. It is not so expected for larval-stage duration, since this period, like fecundity, can readily be affected by certain environmental factors. Larger coefficients of variability for the larval period than for the pupal period are also in keeping with past findings.

ANALYSIS OF PIGMENT DIFFERENCES IN *Tribolium*

The problem.—Tribolium confusum and *T. castaneum* of roughly comparable age appear to be approximately the same color when examined with the unaided eye. The recessive ebony mutant of *T. confusum*, as mentioned at the beginning, is readily distinguished from the type form of either species by its darker body color. As soon as we had established the fact that this was, indeed, a single gene mutation recessive to the normal condition, our interest shifted to the nature of the character which we were studying. It seemed plausible to assume that the similar body color of the type forms of both species was due to the

same quantity of the same pigment in both, whereas the ebony mutant of *T. confusum* contained more of this pigment; but it remained entirely possible that qualitatively different pigments (or perhaps a different distribution and granular structure of the same pigment) were involved. This is the problem reported in this section.

Materials and methods.—Although no direct information to our knowledge is available for *Tribolium*, studies of related forms suggest that we are probably dealing with a melanin pigment similar to that found in the skin and hair of mammals and in the feathers of many birds. Despite a great deal of intensive study, the chemistry of the melanins has remained elusive, and many of the most basic questions related to the formation of this type of pigment are unanswered. Most theories postulate the conversion of a colorless chromogen (tyrosine or some related compound) to a colored substance through one or several chains of oxidative reactions. Some workers believe that the darker melanin pigments are more highly oxidized than the lighter ones and that the process of pigment formation involves an increase in particle size with further oxidation. Others consider some of the lighter-colored pigments (e.g., red) to be more highly oxidized than black. Adherents of the first hypothesis have demonstrated that artificial chromogens which produce what appears to be melanin pigment pass through reddish, then brown, and, finally, black stages as oxidation and increase in particle size proceed and that black pigments are less readily soluble in dilute alkalis than the reds.

TABLE 5

BIOMETRIC CONSTANTS FOR THE RATE OF LARVAL AND PUPAL DEVELOPMENT OF HOMOZYGOUS-DOMINANT AND HOMOZYGOUS-RECESSIVE FORMS

Genotype	Larval Period				Pupal Period			
	Mean Length (Days)	Standard Deviation (Days)	Coefficient of Variability (Per Cent)	n	Mean Length (Days)	Standard Deviation (Days)	Coefficient of Variability (Per Cent)	n
EE males	19.12±0.49	2.32	12.1	10	6.47±0.10	0.44	6.8	9
EE females	17.70±0.30	1.62	9.1	13	6.21±0.09	0.51	8.2	13
EE both	18.31±0.30	2.12	11.6	23	6.32±0.07	0.46	7.3	22
ee males	19.15±0.45	2.13	11.1	10	6.57±0.04	0.17	2.6	7
ee females	18.89±0.22	1.99	10.5	18	6.50±0.05	0.33	5.1	17
ee both	18.98±0.22	1.74	9.2	28	6.52±0.04	0.32	4.9	24

Proponents of the second concept have shown that further treatment of some of these black artificial melanins with oxidizing agents produces a reddish product. Comparisons of absorption spectra reveal that red melanin absorbs more light at the blue end of the visible spectrum and less at the red end than do solutions of black melanin. Further treatment of the black with oxidizing agents, or long periods of standing, produces a shift toward the red type of spectrum. This, however, may be due to the size of the particles in solution and to the subdivision of the larger black particles on standing or receiving special

treatment. No clear indications of the nature of these differences exist to date. Recent papers by Figge (1940), Bohren, Conrad, and Warren (1943), Baker and Andrews (1944), and Ginsburg (1944) contain a review of the literature, as well as data bearing on the question. Tests of the oxidative capacities of pigment-producing cells made with the guinea pig by Russell (1939) indicate that those producing black pigment may have a higher capacity to oxidize dopa (dihydroxyphenylalanine) than those that produce red. Ginsburg (1944), using skin extracts prepared from similar materials, obtained an even greater differential in the same direction. He was able to demonstrate an active substance correlated with the degree of red or yellow pigmentation in the guinea pig but was unable to demonstrate a similar substance for black or brown phenotypes.

Schuurman (1937) presented data for brown and nearly black specimens of *Tenebrio molitor*, which is in the same family as *Tribolium*, that led him to conclude that the differences in pigmentation which he was studying depended upon two different enzymes. The question of the nature of the differences of the end products remains as unanswered here as it did in the studies previously cited.

The foregoing illustrate some of the difficulties involved in the work. Spectrophotometric data are subject to various interpretations, and our inadequate knowledge of the chemical nature and molecular structure of melanin (melanins?) makes its impossible to give absolute quantitative comparisons of amounts of pigment extracted from any natural source. Relative concentrations of pigments having similar absorption spectra may be easily determined, but the nature of the differences between these pigments and pigments having spectra which differ as described above is impossible to state at this time. Such differences may be due to the size of the particles in solution, which may, in turn, reflect the mode of aggregation or deposition of the pigment granules in the animal; or they may depend upon some other quantitative difference. Certainly, no quantitative comparisons are justifiable where the absorption spectra are not identical.

In the work to be reported here the absorption spectra of the pigment solutions are determined in the Evelyn photoelectric colorimeter (Evelyn, 1936), using ten wave-lengths in the visible spectrum and plotting the logarithms of the optical densities observed at each wave-length against the wave-lengths themselves.[5] Where the curves so obtained are parallel, relative concentrations can be estimated by comparing the optical densities at a given wave-length, since these are proportional to the amount of chromogen contained in a given volume of solution. A more detailed description of the methods and principles involved has been given in a previous paper (Ginsburg, 1944).

Since one of the major considerations attending the extraction and analysis of the pigment is quantitative accuracy, it is necessary to determine whether all of the pigment can be extracted by using a solvent in which it is soluble but which will not attack other portions of the integument appreciably. This can be done by comparing the concentration of melanin in solutions obtained by this method with the concentration from the same number of beetles similarly treated but where the chitin is first partly removed by acid hydrolysis. The problem of sepa-

[5] We express our thanks to Dr. E. A. Evans, Jr., of the Department of Biochemistry of the University of Chicago, for permission to use the photoelectric colorimeter.

rating the chitin from the melanin is easily resolved by the solubilities of the two substances. Chitin is soluble in concentrated acids; melanin, in dilute alkalis.

Tribolium melanin may be extracted completely enough for quantitative analysis without first hydrolizing the chitin. The beetles simply are washed, dried between layers of filter paper, and refluxed for 2 hours in 0.2 N potassium hydroxide. Preliminary experiments, during which 2-cc. aliquots are drawn from a 20-cc. sample at ½-hour intervals, establish the fact that extraction is complete in 2 hours. Colorimetric assay shows that, with five independent samples, the solutions become progressively darker until 1½-hours after refluxing is begun. The 2-hour reading is the same as the 1½-hour reading for all these samples; hence this period is more than adequate. Prolonged readings demonstrate that no appreciable change occurs in the solutions if refluxing is continued up to 6 hours or if the solutions are kept in the dark overnight. Twelve imagoes, each 30 days old, are used to prepare every 10-cc. sample reported in the final experiments.

Where attempts are made to remove the chitin before extracting the pigment, less melanin is obtained than with the previous method. This may be due partly to manipulative loss during filtration, but there are some indications that melanin may be actually destroyed or changed during the process. For example, after allowing the beetles to stand for 10 days in concentrated sulphuric acid, a very dark-brown solution is formed. This is filtered through shredded asbestos in a Gooch crucible (a method which Ivaska [unpublished] found adequate to retain the pigment in the filter) and the absorption spectrum of the filtrate is determined (Fig. 2). This filtrate probably contains a variety of substances, since it absorbs light erratically throughout the visible spectrum.

If the asbestos filter, which supposedly contains the melanin, is refluxed for the standard period in 0.2 N potassium hydroxide, the resulting spectrum (Fig. 2) is not at all typical of melanin solutions (Fig. 4). This curve is the type

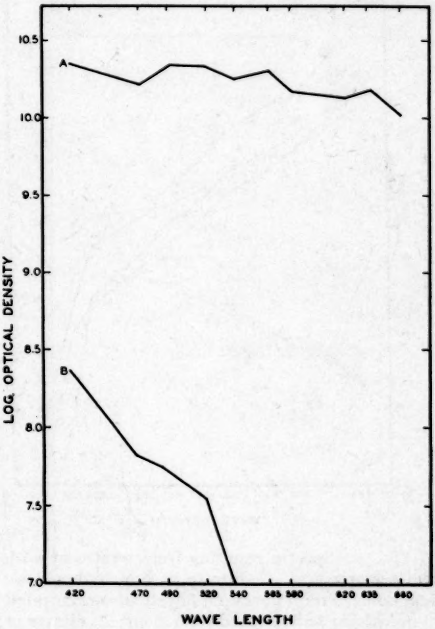

FIG. 2.—Effect of treatment with sulphuric acid on the absorption spectrum of the pigment extract. *A*, filtrate of acid hydrolysate; *B*, residue from above refluxed in potassium hydroxide.

given by solutions obtained by refluxing the beetles in hydrochloric acid (Fig. 3) for periods of 1–2 hours, suggesting that prolonged treatment with sulphuric acid has altered the melanin in some way.

Melanin pigment can be recovered after refluxing the beetles in hydrochloric acid and filtering through shredded asbestos. Here the absorption spectra of the filtrates (Fig. 3) show that no melanin comes through the filter, while that

determined from solutions prepared by refluxing the filter in 0.2 N potassium hydroxide gives a typical melanin absorption spectrum. The quantity of pigment so obtained, however, is somewhat

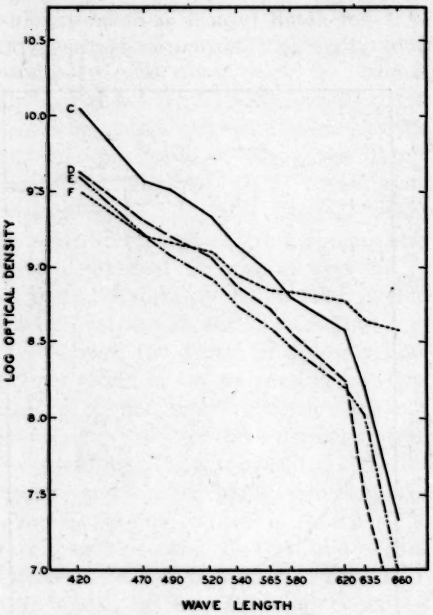

FIG. 3.—Spectra resulting from treatment with hydrochloric acid. *C*, filtrate of 6 N hydrochloric acid refluxed for 2 hours; *D*, filtrate of concentrated hydrochloric acid refluxed for 1 hour; *E*, filtrate of 6 N hydrochloric acid refluxed for 1 hour; *F*, filtrate of 6 N hydrochloric acid refluxed for ½ hour.

below that observed when the step of acid hydrolysis is omitted. Since no pigment is detected in the filtrate, it is possible that refluxing the beetles for as short a period as 2 hours in hydrochloric acid also alters melanin pigment. This view is enhanced by the fact that the spectrum obtained after refluxing for only ½ hour in 6 N hydrochloric acid (Fig. 3) betrays the presence of some melanin in the filtrate. No such traces are detected in any of the samples refluxed for longer periods, nor is the quantity of pigment extractable by direct treatment of the beetles with potassium hydroxide ever realized when acid hydrolysis has first been used. These data, coupled with the fact that prolonged treatment with concentrated sulphuric acid leaves no pigment whatever in the filter, suggest that something other than loss by manipulation is involved.

The final technique calls for the preparation of solutions of melanin pigment by adding 10 cc. of 0.2 N potassium hydroxide to each sample of twelve 30-day old imagoes and refluxing for 2 hours.

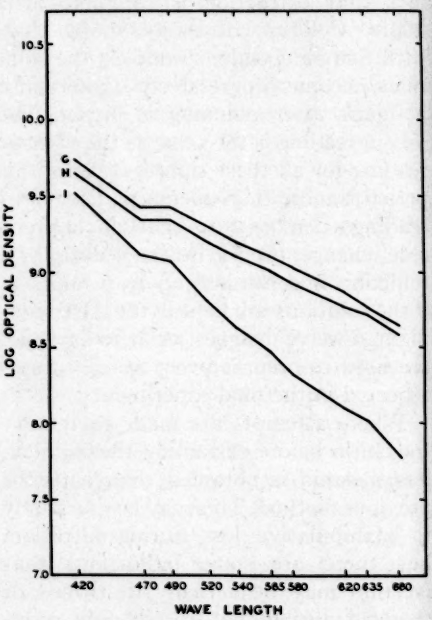

FIG. 4.—Spectra of some natural melanins in potassium hydroxide. *G*, melanin from black guinea pigs dissolved in potassium hydroxide, mean of eight cases; *H*, melanin from potato skins dissolved in potassium hydroxide; *I*, melanin from red guinea pigs dissolved in potassium hydroxide, mean of three cases.

Ten such samples were run for *T. castaneum* and ten for *T. confusum*. Nine were prepared from the ebony mutant of *T. confusum*. Each solution was made up

to 3.5 times its original volume by diluting with potassium hydroxide, and absorption spectra were obtained for each sample separately in the Evelyn photoelectric colorimeter. Identical spectra are summarized in the accompanying figures by a single curve representing the average of the group. Concentrations are expressed as optical densities, since no method for estimating absolute quantities of melanin pigments exists and since it can be shown that the optical density is proportional to the amount of chromogen contained in the solution. A comparison of these optical densities, therefore, provides a means for estimating relative concentrations of pigment where the absorption spectra of the solutions being tested show the same shape and slope when graphed by the method used here. All quantitative comparisons are made at 420 mμ because this represents the absorption peak in the range of wavelengths explored in this study. Melanins show no well-defined absorption band in any area of the spectrum and could consequently be compared at various wavelengths. The wave-length used here is merely an arbitrary one selected for convenience and because other data obtained in this laboratory were derived in the same way.

Major results.—The slopes of the curves (Fig. 5) obtained from the absorption spectra of *T. confusum* and *T. castaneum* by the methods discussed above are different; hence no quantitative comparisons can be validly made between the two species. Similar differences have been reported for black and red guinea-pig melanin by Baker and Andrews (1944) and by Ginsburg (1944). Bohren, Conrad, and Warren (1943) have observed the same phenomenon in black, buff, and red varieties of many breeds of domestic poultry. We have already discussed the interpretation of these differences in slope and indicated that, though it is possible that they reflect a difference in the molecular structure of the pigments, it is also possible that they are produced by differences in physical state. No final interpretation can be given until more critical data are collected and evaluated than exist at present. All that we actually know is that our laboratory stocks representing these two species differ in an aspect of their pigmentation which is reflected by the steepness of their light absorption curves.

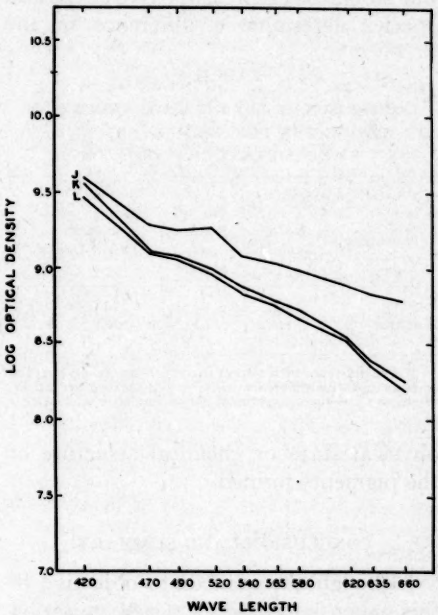

FIG. 5.—*Tribolium* melanin in potassium hydroxide. *J*, *T. castaneum; K*, *T. confusum*, normal; *L*, *T. confusum*, ebony. Each curve represents the mean of ten samples, variously diluted.

The type form and ebony mutant of *T. confusum* (Fig. 5) have the same kind of pigment; i.e., solutions prepared from these phenotypes have identical slopes when their light absorption properties are graphed. They may be, therefore,

validly compared in a quantitative fashion (Table 6). Such a comparison reveals that the ebony mutant contains 17.5 per cent more pigment than the type form from which it has presumably descended. The genetic difference in this instance determines a quantitative change in the amount of pigment deposited. The genetic differences between our laboratory representatives of the two species determine a difference in the physical state or chemical structure of the pigments formed.

TABLE 6

COMPARISON OF THE RELATIVE AMOUNTS OF PIGMENT IN THE NORMAL FORM AND EBONY MUTANT OF *T. confusum*

Phenotype	No. of Samples	No. of Beetles per Sample	Mean Optical Density*
Ebony.....	9	12	0.341±0.011
Normal.....	10	12	0.281±0.014

* The difference with respect to optical density is 0.060. The odds against this difference being due to chance are 46 : 1, where 20 : 1 is the conventional border line of significance.

CONCLUSIONS AND SUMMARY

Although the immediate objective of this paper is to describe a new mutation for *T. confusum*, discuss the mechanism of its inheritance, and analyze certain of its physiological effects, the larger objective is to lay a background of information about a mutant type that can be used in studies of intra- and interspecies competition in laboratory populations. "Ebony" is admirably suited for such investigations because of its obvious visual difference from normal type and because its reduced fecundity places it at a slight selective disadvantage when in competition with EE beetles. As pointed out at the beginning, such experiments are now in progress, and reports may be anticipated.

The essential findings of the present study are these:

1. A gene, named "ebony," is described which, when homozygous, causes the imago of *T. confusum* to appear nearly black to the eye instead of the red-brown color characteristic of the normal type.

2. Ebony is inherited as a single, recessive, autosomal gene.

3. The fecundity of ebony imagoes is approximately 10 per cent lower than that of normal type imagoes. This is a statistically significant difference. There is no significant difference between homozygous normal and heterozygous beetles in respect of fecundity.

4. The ebony gene has no significant effect on egg fertility (hatchability).

5. The rate of larval and pupal development is not affected by the ebony gene.

6. With respect to the studies on pigmentation the following conclusions are reached:

a) The absorption spectra given by solutions of melanin pigment prepared from *T. confusum* are different from those of *T. castaneum*, at least for the stocks maintained in our laboratory. These differences may be due to qualitatively different molecular structures or to disparities in the physical state of the melanin in these two species.

b) The ebony mutant of *T. confusum* possesses pigment which yields absorption spectra identical with those given by pigment from the type form but contains approximately 17.5 per cent more melanin than the latter.

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THE GOLDEN HAMSTER (CRICETUS AURATUS) CARE, BREEDING, AND GROWTH[1]

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THE golden hamster, although only recently introduced into the United States, has attracted considerable attention in many laboratories. Questions have arisen concerning the suitability of this rodent as a general laboratory animal, its availability, habits, and general care.

In 1942 a single pair of animals was made available to this laboratory. Their two litters of 4 and 11 young, respectively, with 15 young females purchased from a hamster-breeder, formed the source for an actively breeding colony from which approximately 1,500 animals have been taken for various experimental studies on reproduction, biology of sex, and studies on internal secretion. Several of these problems are under investigation at the present time under the general direction of Professor Carl R. Moore. Because of numerous inquiries concerning the maintenance and production of such a colony, it appears desirable to record some of our general experiences along this line.

ORIGIN OF THE STOCK

The golden hamster is a rodent native to Syria with an apparently restricted geographical distribution. Bruce and Hindle (1934) trace the entire laboratory stock of this species in the British Isles to a single litter, dug from an 8-foot burrow near Aleppo, Syria, in April, 1930, by Mr. I. Aharoni. The young of this litter, maintained at the Hebrew University in Jerusalem, produced the first-known litters born in the laboratory. From offspring of this stock two pairs were carried to England by Dr. Saul Adler, of the Microbiological Institute, Jerusalem, and presented to Professor Edward Hindle in 1931. This is substantiated in a letter of February, 1941, from Professor Hindle, of the University of Glasgow, to Mr. Henry Bergman, a hamster-breeder of Springfield, Missouri.

The American laboratory stock of the golden hamster is probably of the same genetic strain as the British stock, since in a letter to Dr. Moore in 1942, Dr. G. H. Faget, of the United States Public Health Service, Carville, Louisiana, states that in 1938 a shipment of hamsters was received at their laboratory from Dr. S. Adler of Palestine and that in the same shipment animals were sent to Western Reserve University. Our own original pair derives from the United States Public Health Service stock via the Purina Mills of St. Louis, Missouri. The 15 females which were bred into our

[1] This investigation has been aided by a grant from Armour and Company and from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

colony issued in a roundabout manner from the Western Reserve stock. We have been unable to learn of any American stock that cannot be traced back to the original shipments received in this country from England in 1938 by the two above-mentioned laboratories.

HOUSING AND FEEDING

Our colony has been housed in a basement room with relatively poor ventilation and temperature control. Wire-mesh cages of sizes varying from 35 × 25 × 15 inches to 15 × 12 × 12 inches have been used. Wooden pens proved to be impractical, as the animals rapidly gnaw their way to freedom. Wood shavings or sawdust are used to cover the cage bottoms, and excelsior is provided for nest material.

Groups of 8–10 animals may be housed together in the larger cages, especially if they are members of the same litter. Because they are vicious fighters, it is often difficult to place adults from different litters in the same cage, even if they are of the opposite sex. It has frequently been necessary to place incompatible animals separately in small cages.

Diet may be widely varied; but in the ordinary colony maintenance, grains such as corn, oats, and wheat, mixed with Purina fox-chow and dog-chow checkers, proved to be satisfactory. Water is essential for the maintenance of weight; but it has been found that fresh vegetables, such as carrots, cabbage, or lettuce, form an adequate source of fluid intake. Since these animals are largely nocturnal, they feed during the evening. If vegetable food is placed in the cages early in the day, much of it will be wasted through drying. All wastage cannot be eliminated, because the hamster has a propensity for storing food in corners. Supplements of milk, up to 4 ounces a day per animal in the case of pregnant or lactating females, have been found to be advantageous.

BREEDING

The oestrus cycle of the female hamster is short and has been indicated by Deanesly (1938) to have an interval of 4 days. The vaginal-smear technique has not been used by us to determine the cycle, since the presence of lateral pouches in the lower vagina, containing quantities of constantly cornified epithelium, makes difficult and hazardous the interpretation of vaginal smears taken in the routine manner. Since this work was first started, Peczenik (1942*a*) has studied the cyclic vaginal pattern through the use of a loop inserted high in the vagina in such a manner as to avoid contamination from the lateral pouch plugs. Even more recently Kupperman, Greenblatt, and Hair (1944) have employed vaginal-lavage technique to observe the same phenomenon. Both methods appear to be satisfactory.

In this laboratory, cursory observations were made in an attempt to determine cyclic variation in nocturnal activity by means of activity-recording cages. It has earlier been shown that female rats are more active during oestrus than at any other period of the cycle, with a falling-off in activity during postoestrus and metoestrus and a gradual rise beginning with pro-oestrus. These cycles are not unmodifiable and are subject to some degree of variation. Although in our study only a selected group of hamsters was used, results over a 40-day period showed significant changes in activity of a cyclic nature with only slight variations in length of period. The activity of two representative females is shown in Figure 1. In this graph it should be noted that female 31-A, at a peak of activity, recorded 17,000 revolutions in

one 24-hour period, which means that this animal traveled a distance of 1.0008 miles. The greatest number of revolutions recorded for a given female during a 24-hour day is 33,249, or 1.9416 miles (not shown in this graph). From the regularity of these graphed cycles we feel that it can be stated that an activity of a definite cyclic nature is apparent, probably correlated with the 4-day physiological cycle of the female hamster.

In the breeding of these animals, if timed pregnancies are not essential, a male and female may be caged together until the female is obviously pregnant. However, in many cases a male and female caged together for long periods of time may not produce litters. When repaired with a strange animal, each may demonstrate its fertility. For ordinary breeding purposes in this colony, males and females were caged apart until those times at which it was desirable to have new litters. Then, in the evening, i.e., any time after 6:30 P.M., a male was introduced into a cage holding one or several females; and if a sexual response occurred, the reacting animals were left together overnight. Matings have not been recorded in the daytime, the earliest known response being at 6:17 P.M. on a winter evening. If no response was obtained, it was assumed that the female was not in heat, and the male was removed. The mating response is usually immediate if the female is in heat, and the pattern of the response is similar to that of rats. The female exhibits pronounced lordosis; but copulations are repeated many times and over a much longer period than in the rat. We have found that the female response cannot always be definitely associated with oestrus, since these animals not infrequently exhibit mating responses during pregnancy and have even been observed to copulate up to 1 day prior to parturition.

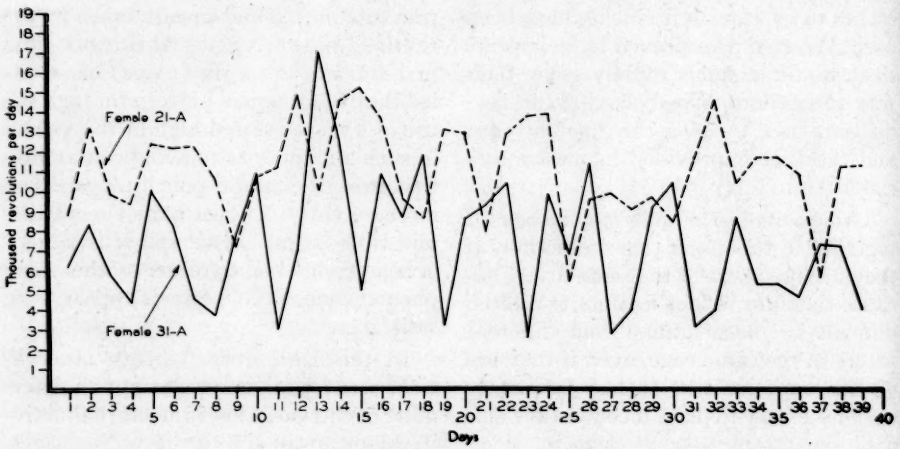

FIG. 1.—Graph of cyclic activity of 2 female hamsters over a 40-day period

In our laboratory, matings have occurred throughout the year, and litters have been recorded in every month. The seasonal cycle of breeding suggested by Bruce and Hindle (1934), Deanesly (1938) and Peczenik (1942*b*), working in England, has not been in evidence. Rather than the presence of a winter slump from October to May, our most intensive

breeding period coincides with that time; i.e., under controlled breeding our colony produced 92 litters between November, 1942, and May, 1943, as contrasted with 19 litters delivered between June, 1943, and October, 1943.

The three principal factors of greatest importance in successful breeding in our experience have been (*a*) diet, (*b*) temperature, and (*c*) age of females.

The general diet, as outlined, has proved adequate for continuous breeding, and no special additions have been employed. One breeding slump of a few months occurred when insufficient amounts of food were made available to the animals. This not only led to a serious drop in the numbers of litters, but in many animals it was also reflected in a 20 per cent body weight loss during the course of two months. Not all animals were affected, but in some of the males the testes were greatly reduced in size. The mere increase in amount of food very soon restored breeding to normal proportions and also returned body weights to normal.

The room temperature of our breeding colony was maintained at 70° F., or above. A drop in room temperature to 45° F. frequently caused the animals to go into a hibernating state. They go into a deep sleep; the body becomes quite rigid; and body temperature falls below normal. In all cases the animals can be revived after warming for a period of 15 minutes. Those not in an actual hibernating state may be quite lethargic. Under these conditions the animals remaining active may kill their lethargic or comatose cage-mates. Mothers may consume entirely a large litter which is hibernating. Matings in these reduced temperatures are infrequent and largely unsuccessful.

Our recorded data thus far suggest that female hamsters cease to breed at about 1 year of age. No special means have been used to determine whether females older than a year can be stimulated to reproduce, but the fact remains that among two hundred litters only a single litter has been recorded from a female older than 10 months; i.e., one female littered at an age of 11 months and 3 weeks. Males, on the other hand, have continued to sire litters when more than 2 years of age. Insufficient data are available to suggest the average life-span of this species.

A minor factor of suggested sterility, perhaps intensified in this colony through close inbreeding, has been noted, as well as a factor for runtiness. These are not at all frequent, but in some litters runts are very apparent. Breeding of these particular animals has not been encouraged; but in some instances where breeding has occurred, it has been noted that the larger siblings produce young considerably earlier than the smaller ones.

The sterility factor mentioned above may be illustrated by the two following females:

Female 4-A.—Mating response for months was very irregular, and intervals between copulations ranged from 8 to 15 days. This animal raised two litters before she was 6 months of age. During the following 8 months she mated frequently but failed to become pregnant. There are definite recorded entries for ten copulations during this period to healthy, potent males; and the presence of motile spermatozoa was demonstrated 15 minutes after the first mating response. Other unrecorded matings probably occurred, but no further pregnancies resulted.

Female 11-A.—This animal produced her first litter at 2 months of age. Following this period, her record shows nine dated copulations, with motile sperm demonstrated; but only once, in the third month, did a pregnancy follow, with gestation running for 18 days, and a final delivery of abnormal, dead young.

The total number of litters a female will produce has not been determined, since matings have been restricted to the

number necessary to maintain the colony in an active condition and to provide animals for experiments. However, four or five litters in as many months for a single female have not been unusual, and we presume that many additional litters are possible with intensive breeding.

GESTATION, LITTER SIZE, SEX RATIO, AND SEXUAL MATURITY

One element of particular interest to investigators in this laboratory was the alleged short period of gestation. It was anticipated that periods considerably shorter than the 21–22-day period of rats and mice might provide special advantages for certain studies on sex differentiation. In regard to the early development of the hamster, Graves (1943), in his report on the embryology, notes especial rapidity of development up to the establishment of the intermediate cell mass. He states that this animal develops faster than any known placental mammal and fully as rapidly as the opossum. Bruce and Hindle (1934) and Deanesly (1938) have reported the gestation period to be approximately 16 days.

In order to determine accurately the length of gestation, as well as to obtain a definite series of embryonic stages, witnessed copulations were strictly employed in our work. Length of gestation is expressed as the time from first copulation to delivery. Since Deanesly (1938) states that ovulation follows within a few hours after mating, time of first copulation deviates but little from the actual length of time since fertilization. In a series of 28 females the mean period from first copulation to delivery proved to be 381 ± 1.0 hours, or 15 days and 21 hours.

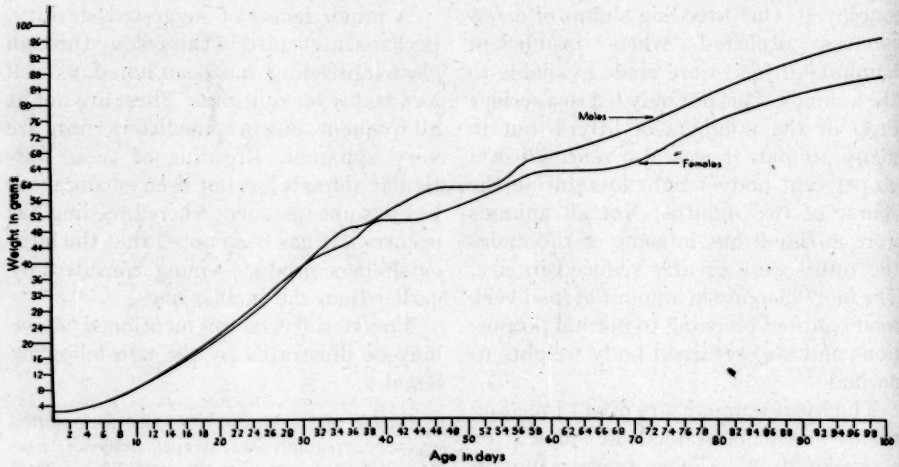

FIG. 2.—Graph showing comparative growth curves for male and female hamsters from birth to 100 days of age.

Definite records in the case of 107 deliveries show that the number of young per litter ranged from 2 to 15, with a mean litter size of 6.93 ± 0.23 and a standard deviation of ± 2.414. The sex ratio for the colony between April, 1942, and October, 1943, was 338 males to 325 females, with a deviation 0.505 times as great as the standard error.

When a young litter is to be used for experiment, some precaution is advisable, since frequently the mothers may devour their young. Our experience has shown, in general, that young hamsters require more careful handling than young rats. However, they can be safely handled from the time of birth. Frequent handling of pregnant females in order to tame them before delivery, removal of the mother from the nest prior to removal of young, and their return to the nest before the mother have all been utilized as precautionary measures. Where but a few of the very young may be used for experimental purposes, removal of the mother until all young are returned is advisable. A bleeding animal should never be replaced immediately—a good precaution also in the case of adults—since other cage members may kill such animals. Females should not be permitted to deliver in cages where others are present, since either they or the other adults will frequently kill the offspring. A male paired with the female may kill the litter; but he, in turn, may be killed by the female after delivery has been completed.

TABLE 1

DATA ON GROWTH OF MALES AND FEMALES

Age in Days	Males		Females		Age in Days	Males		Females	
	No. of Cases	Mean Weight (Gm.)	No. of Cases	Mean Weight (Gm.)		No. of Cases	Mean Weight (Gm.)	No. of Cases	Mean Weight (Gm.)
Birth	39	2.179	40	2.225	33	25	44.760	24	42.125
1	44	2.454	41	2.585	34	30	46.400	39	42.179
2	37	2.837	30	3.033	35	23	49.565	30	44.400
3	47	3.695	53	3.641	36	17	51.529	22	48.227
4	45	4.133	46	4.130	37	51	45.705	37	46.513
5	31	4.709	34	4.852	38	23	49.478	24	47.583
6	32	5.406	26	5.307	39	17	57.411	27	50.185
7	33	6.212	42	6.166	40	20	53.300	33	54.575
8	21	6.857	17	7.058	41	28	55.357	40	53.825
9	34	8.058	38	7.763	42	25	52.480	59	47.813
10	44	7.772	51	7.764	43	31	53.709	39	50.025
11	27	8.740	21	9.476	44	21	56.904	30	49.000
12	38	10.421	35	10.171	45	21	61.095	40	49.900
13	33	12.787	36	12.722	46	19	60.368	34	51.205
14	41	12.853	46	13.674	47	25	59.040	31	55.677
15	32	14.062	33	15.154	48	24	61.750	27	58.481
16	40	15.525	45	15.911	49	35	57.342	56	53.660
17	46	16.239	50	16.340	50	35	60.685	32	55.468
18	29	21.655	26	19.230	51	27	57.296	21	55.476
19	39	21.205	42	21.357	52	13	64.230	33	54.606
20	26	23.000	24	23.375	53	23	56.913	47	56.553
21	36	25.611	30	24.466	54	25	61.360	15	61.066
22	35	24.475	38	25.500	55	17	63.941	27	65.259
23	28	28.071	31	28.096	56	36	63.638	62	60.467
24	29	26.379	23	30.130	57	29	66.137	28	53.678
25	36	30.472	39	30.076	58	21	68.619	18	63.277
26	29	33.000	41	31.585	59	22	72.636	21	72.523
27	27	33.259	29	34.586	60	38	64.657	21	63.428
28	26	37.807	21	35.714	61	24	66.916	9	66.222
29	52	37.462	60	37.166	62	26	67.807	16	66.812
30	27	39.555	32	37.625	63	43	68.976	38	65.184
31	35	42.342	33	40.666	64	22	70.727	13	67.769
32	24	44.250	26	43.154	65–100	696	86.139	393	78.609

Postnatal development is rapid. The mean weight at birth is 2 gm., and it increases at approximately 1 gm. per day for the first 2 weeks. Figure 2 presents comparative graphs of male and female growth curves, which were determined in the following manner. From birth to 8 days of age, the mean weights were plotted on each day. From 8 to 35 days of age, a 3-day running average was computed, and the points were located on the mean day for each 3-day block. From 35–65 days of age, mean weights were determined for 5-day blocks and were centered on the average day for each block. Since the growth curve tends toward an asymptote beyond 65 days of age, all the weights from this age to 100 days were lumped together, and the mean weight of the group was centered on the mean day. In Table 1 are recorded the actual data employed—i.e., age, number of cases, and the average weights obtained for each day. Deviations from the means and coefficients of variability have been determined for representative days and are recorded in Table 2. Figures were not included in the graphs for animals over 100 days of age, since weights at older ages would obviously be modified by increased fat deposition. From data on individuals over 100 days of age (100–400 days) the average weight of males is found to be 100.4 ± 1.003 gm. (354 cases) and of females, 95.4 ± 0.95 gm. (373 cases).

At birth the young are deep pink, but they begin to show black skin pigment on the dorsal surface at 60 hours after birth. By 72 hours the backs are deeply pigmented. Teeth are evident at birth; and the young eat hard food by the eighth day, prior to the opening of the eyes, which occurs between the fourteenth and the fifteenth day. Young females display an opening of the vaginal orifice very consistently at a weight of 8 gm., which is attained from the eighth to the fourteenth day of life. Rupture of the vaginal membrane shows a more accurate correlation with weight than with age. The opening of the vagina in this animal appears to have a different significance from this same phenomenon in the rat or mouse, since it has been pointed out that the first spontaneous oestrus does not occur until about the thirty-second day (Peczenik, 1942*a*). Kupperman, Greenblatt, and Hair (1944) state that vaginal introitus is formed between the tenth and fifteenth day of life but

TABLE 2

GROWTH DATA TREATED STATISTICALLY

Age in Days	No. of Cases	Mean Weight (Gm.)	Standard Deviations	Coefficients of Variability
	Males			
Birth	39	2.179	.5074	23.285
4	45	4.133	.6263	15.153
10	44	7.772	2.178	28.023
19	39	21.205	4.543	21.424
25	36	30.472	5.775	18.951
29	52	37.462	5.394	14.398
34	30	46.400	8.147	17.558
40	22	53.091	8.017	15.100
45	21	61.095	12.054	19.729
50	35	60.685	11.192	18.442
56	36	63.638	9.57	15.040
60	38	64.657	8.87	13.720
65–100	696	86.139	14.354	16.664
100+	354	100.38	18.87	18.79
	Females			
Birth	40	2.225	.4797	21.56
4	46	4.130	.7498	18.155
10	51	7.764	1.882	24.240
19	42	21.357	5.093	23.847
25	39	30.076	4.765	15.843
29	60	37.166	4.603	12.368
34	39	42.179	6.699	15.882
40	33	54.575	7.933	14.536
45	40	49.900	12.091	24.234
50	32	55.468	11.923	21.495
56	62	60.467	11.749	19.430
60	21	63.428	10.473	16.511
65–100	393	78.609	17.136	21.672
100+	373	95.4	18.4	19.28

that corpora lutea formation and cyclic changes in the vagina are not initiated until the thirty-third to the thirty-eighth day of life. In our colony we also found that the capacity for reproduction does occur very early—perhaps earlier than for any other known mammal. Copulations have been observed in litters caged together from birth by the forty-third day, and delivery has been observed by the fifty-ninth day. Infertile copulations have been recorded as early as the thirtieth day. The range in age of females at first littering is 59–90 days, and in 35 definitely recorded cases the mean age at first delivery is 73.6 ± 1.50 days, with a standard deviation of ±8.99. Males have been seen to copulate with their mothers by the forty-fifth day, but the age of first appearance of spermatozoa has not yet been determined by us. Since our observations, however, Kupperman, Greenblatt, and Hair (1944) have noted that "spermatogenesis commences about the 35th day of life."

SUMMARY AND CONCLUSIONS

The general features of colony maintenance of the golden hamster have been studied in a group of approximately 1,500 animals over a 2-year period. As a result of these studies, the following conclusions have been drawn:

1. These animals should be housed in metal cages and provided with a varied diet of grain and fresh vegetables.

2. Activity of a 4-day cyclic nature has been observed in the females. This may correspond to the oestrus cycle.

3. Matings usually occur at night. No seasonal distribution of litters is apparent. Principal factors affecting breeding are (*a*) diet, (*b*) temperature, and (*c*) age of females.

4. Length of gestation period (15 days and 21 hours), determined from time of copulation to delivery, agrees with that found by earlier workers.

5. Postnatal development is rapid. Eight-day-old hamsters are able to get along without maternal care, in spite of the fact that their eyes do not open until the fourteenth to fifteenth day.

6. The vagina opens when the female attains a weight of 8 gm.; this may occur any time between the eighth and fourteenth day of age.

7. Delivery of litters may occur at an age of 59 days, but the mean age at first littering (35 cases) is 73.6 days.

8. Males may copulate by the thirtieth day, but fertile matings have not occurred until the forty-third day.

9. The mean litter size, as determined from 107 cases, is 6.93 ± 0.23 animals with a standard deviation of ±2.414.

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STUDIES ON METABOLISM OF TAENIA TAENIAEFORMIS[1]

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THE biology of those internal parasites with finely adjusted host specificity invites attention to physiological problems which are closely integrated with those of the respective host and yet which are peculiarly unique.

In recent years several reviews upon the subject of helminth physiology have appeared (von Brand, 1934; McCoy, 1935; Wardle, 1937*b*). Also, reviews of a more general nature contain considerable material on the physiology of parasites (Peters, 1936; Hoeppli, 1937; and Hoeppli, Feng, and Chu, 1938).

For the most part, the *in vitro* culture of parasitic forms has encountered serious difficulties. To date, trial-and-error methods have proved about the only means of devising satisfactory culture media. The culture of facultative parasites and of free-living stages of obligatory parasites has been comparatively easy, since these forms are already adapted to the environment outside the host. Parasitic stages present a much more complex problem. The correct combination and sequence of the optima of a large number of factors must be achieved. Knowledge of such factors will be gathered piecemeal, mostly from physiological investigations of each factor separately.

PROBLEM

Inasmuch as many parasites are measurably pathogenic, a complete knowledge of their biology is needed to circumvent their activities most efficiently. Comparatively little is known of the physiology of intestinal and tissue parasites; and, since controlled *in vivo* experiments present obvious difficulties, an attempt has been made to devise methods for the *in vitro* maintenance of some common parasites and to study factors affecting *in vitro* culture as they pertain to metabolic activities. The tapeworm *Taenia taeniaeformis* Batsch, 1786 (Wolffhuegel, 1911), was used in these studies.

MATERIALS AND METHODS

Taenia taeniaeformis is the broad-necked tapeworm, commonly parasitic in the domestic cat. The synonym, *T. crassicollis*, has been rather widely used for this species in the literature. The larval stage is a strobilocercus and is known by the name of *Cysticercus fasciolaris*. It commonly occurs in cysts on the livers of rats and mice. Distribution of this parasite, along with its common hosts is probably world-wide. In 1878 Leuckart investigated certain phases of its life-cycle and came to the conclusion that the larval strobilus degenerated after ingestion by the definitive host and that the adult proglottids arose anew. Joyeux and Baer (1937) essentially confirmed Leuckart's work but found that only the posterior one-half to one-third degenerated.

The intermediate host ingests the eggs in fecal-polluted food. Onchospheres bore through the intestinal wall, are carried by the portal circulation to the liver, and come to lie in capillaries of that organ.

[1] Appreciation is expressed to Professor H. W. Stunkard for aid and direction during the course of the present investigation.

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Bullock and Curtis (1924) reported a larva, which measured about 15 μ in diameter, from a liver capillary 21 hours after the host had been fed infective feces. About the sixth day larvae can be found on the surface of the liver, where they appear as small blisters or vesicles. By the end of 15 weeks the normal larva may reach a length of 5–6 cm., with well-pronounced segmentation and a caudal vesicle. Infective larvae have been secured in slightly more than 7 months, as reported by Bullock, Curtis, and Dunning (unpublished). When the definitive host eats an infected rodent liver, the larvae are freed by the action of digestion and usually come to lie a little anterior to the middle of the small intestine. Eggs appear in the cat feces in 35–42 days. Thus the complete life-cycle of this worm would require a minimum of about 250 days.

This parasite was chosen for study because it presents a number of advantages. Its hosts are well-known laboratory animals, and considerable knowledge is available concerning the anatomy and physiology of their organ systems. The life-cycle of the parasite is well known, and the hosts are easy to infect. The larval stage of this worm is a tissue parasite, and the rodent host develops an immunity against hyperinfection (Miller, 1931, 1934, and 1935 and Miller and Gardiner, 1934). Therefore, the investigator using such material knows that the parasites are all of the same or very closely related feedings and consequently about the same age.

Another factor of great importance is that usually the parasites from the rat liver are sterile. Bullock and Curtis (1924) found 2 cases of staphylococci in testing liver fragments of 20 rats infected with *C. fasciolaris*. In most cases, therefore, the absence of bacteria provides a ready-made feature of control.

Laboratory rats were fed eggs containing infective onchospheres by placing the material far back in the throat with a long medicine dropper. This method was used by Bullock and Curtis (1924). The number of onchospheres per drop of suspension was determined, as excessive infections produce high mortality of the host or, if the animal survived, larvae in "crowded" infections tend to be smaller. Some rats were fed by keeping them without food for 24 hours and then giving them pieces of bread well moistened with infective onchosphere suspension. Laboratory cats were infected by distributing cysts from the rat liver in chopped meat.

To obtain the larvae of *T. taeniaeformis*, rats were killed by ether administration or by a blow on the head. In attempting to prevent the ingress of bacteria, sterile precautions were observed. The abdomen of the rodent was soaked with alcohol, and lobes of the liver with attached cysts were removed with sterile instruments and placed under autoclaved mammalian Ringer's solution, maintained as close to 37.5° C. as possible. Larvae were removed from the cysts, or in a few cases the entire cyst was removed from the liver, below the surface of the sterile solution. These cysts and larvae were quickly transferred to a fresh volume of the Ringer's solution to wash them of blood. They were then placed in sterile media and maintained at 37.5° C. Types of containers used were 50-ml. and 250-ml. Erlenmeyer flasks and 6-inch Pyrex test tubes. Bacteriological procedure was followed in transferring worms from one flask to another, although great care had to be exercised to prevent the worms from coming into contact with the flamed neck of the flask. Cotton plugs were used to close the containers in a number of instances, but usually sterilized rubber stoppers were substituted. These stoppers were treat-

ed after the method of Buchsbaum (1936) and were autoclaved individually wrapped in cheesecloth.

In beginning a study of the physiology of this tapeworm it was deemed advisable to determine how long the animals would survive outside the body of the host. Various nonnutrient solutions were used initially; and, later, media containing simple nutrients were tried. Tyrode's solution was chosen as the base for most of the culture experiments, although occasionally mammalian Ringer's solution was substituted. Tyrode's solution cannot be autoclaved in the final form, but the sodium bicarbonate can be autoclaved as a separate solution and then added to the other autoclaved components (Buchsbaum, 1936). Sometimes disodium phosphate was substituted for the sodium bicarbonate.

In the preparation of more complex media, Difco's tryptone and peptone were used whole; but more often these products were digested with pancreatin and this digest used for amino acid source. Digests of rat and cat tissues were used as amino acid sources in some experiments. These digests were utilized as liquids in some cases, but the usual method was to prepare these amino acid complexes in agar. Then the worms were placed in Tyrode's solution, or its modifications, on top of the agar, so that a gradual seepage of amino acids from the agar would occur. In certain experiments acriflavine (National Aniline and Chemical Company) and the bile salt sodium taurocholate were added to the amino acid–agar complex.

Usually the worms were transferred to fresh media at frequent intervals, but in some cases the medium was unchanged during the course of the experiment.

Activity was the principal criterion for determining the length of life of worms living in artificial media. A mild shaking of the container usually provided sufficient stimulation, although occasionally gentle probing was utilized.

The Winkler method was used for measurements of oxygen consumption. A number of modifications for measuring oxygen in small amounts of fluid have been reported in the literature. Fox and Wingfield (1938) discussed an apparatus suitable for such determinations. In the present study a 30-ml. hypodermic syringe was used for a respiratory chamber. After filling it with the desired amount of fluid and placing the worms in it, the syringe was inverted and any air present was forced out. A needle whose shaft was closed with a rubber stopper was used to close the neck of this syringe. A 10-ml. hypodermic syringe was used for a reaction chamber. For transferring fluid from one chamber to the other a needle with a piece of rubber stopper set midway on the shaft was placed on the inverted respiratory syringe. The plunger of the reaction chamber was completely closed, and then the neck of this syringe was placed over the open end of the needle on the respiratory chamber and held firmly against the piece of rubber stopper on the shaft of this needle so that a tight connection was established. Liquid was forced in to fill the blind space in the neck of the reaction chamber, and the air here was forced out. The amount of fluid to be tested was then forced into the reaction chamber. The necessary amounts of reagents of the Winkler method were drawn into the reaction chamber. The clear yellow solution left after the final reaction was placed in a small Erlenmeyer flask and titrated with 0.001 N sodium thiosulphate with starch as an indicator. The thiosulphate was standardized against iodate according to the directions of Peters and Van Slyke (1932). Two cubic centimeters of solution were found satisfactory for oxygen measure-

ment at each determination; and determinations were made at 10-minute intervals, first in a series in normal mammalian Ringer's solution, followed shortly by a series with the same worms in various concentrations of potassium cyanide in Ringer's. This drug was used since it is a specific poison for the Warburg-Keilin cytochrome system of cellular oxidation. Between and during tests these larvae were maintained at body temperature.

Studies on the reduction of methylene blue were made with standard Thunberg tubes, evacuated of air. A comparison was made between methylene blue reduction in mammalian Ringer's solution and in mammalian Ringer's containing 1 per cent glucose. Measurements of the change in color of the methylene blue were made electrometrically.

Anaerobic studies were made by various methods. One of these is a standard bacteriological technique in which freshly ground meat is placed in the lower half of standard 6-inch test tubes and covered with Tyrode's or Ringer's solution. An anaerobic environment existed at the bottom of such tubes. Another method was the utilization of a sodium thioglycollate medium containing pork infusion solids, peptone, and dextrose (Brewer, 1940). Methylene blue is present in this liquid medium as an indicator. This medium was used in 6-inch test tubes of small diameter. The solution is aerobic at the top, as indicated by the methylene blue, and becomes successively more anaerobic toward the bottom. Consequently, the height of the column of medium can be the determining factor in the amount of anaerobiosis at the bottom of the tube. In some tests an anaerobic condition was approached by placing the worms in test tubes in various media and covering the solution with a layer of Nujol, 35 mm. or more thick. Then, with an allowance of only a minimum of air space for displacement, the test tube was tightly closed with a rubber stopper. After the larvae have utilized the oxygen already present in the medium, they will have to respire anaerobically to survive.

Cat serum was obtained from an anaesthetized cat by means of a sterile citrated syringe, with the blood being drawn directly from the heart. After coagulation in sterile vessels the serum was decanted and placed in sterile ampoules. As further precipitation sometimes occurred, only the serum in the upper part of the ampoule was used, and this was drawn off directly by sterile hypodermic syringe.

Chick embryonic extract was made from eggs of 10 and 12 days' incubation. The embryonic material was aseptically removed, cut into minute fragments, and placed in flasks in Ringer's solution. Only the supernatant fluid was used for experiment.

Surface tension was reduced by the use of bile salts and of hexylresorcinol (S.T. 37). Measurements of pH were made in the La Motte comparator for the most part, but in some later experiments pH papers (Paul Frank) were utilized.

Because of the location of the larvae of *T. taeniaeformis* in the liver, it was considered that uric acid, allantoin, and even urea might have some effect on the metabolism of these worms. Robinson and Wilson (1939) showed the relationship of concentration of the enzyme urease with the reorganization occurring in the pupa of *Phormia regina*. Urease is specific for urea, and the concentration was greatest between 30 and 80 hours of pupal life, falling sharply at the end of the pupal period. Such a correlation might indicate that urea has a significant part in this reorganization process. Matthews (1936) has pointed out that

urea, which penetrates cells readily, has powers of dissolving proteins, starches, and other substances. Therefore it could conceivably act on glycogen. He also pointed out that allantoin may be the curative principle in the healing of certain wounds—a regeneration process that may be akin to growth.

TABLE 1

A COMPILATION OF THE RESULTS OF EXPERIMENTS IN WHICH LARVAE AND ADULTS OF *T. taeniaeformis* HAVE BEEN MAINTAINED ALIVE

SEC. A

MEDIA WITHOUT AMINO NITROGEN SOURCES

Larvae

Medium	Survival (in Hours)
Tyrode's solution (nonsterile)	324
Tyrode's solution plus 0.01 per cent phenol	324
Tyrode's solution	156*
Tyrode's solution	50*
Modified Tyrode (double sodium bicarbonate)	384
Modified Tyrode (disodium phosphate replaced sodium bicarbonate)	293
Modified Tyrode (double sodium bicarbonate) plus 0.01 per cent urea	161
Modified Tyrode (double sodium bicarbonate) plus 0.01 per cent urea	192
Tyrode over plain agar (2 per cent)	146
Mammalian Ringer's solution	161
Mammalian Ringer's plus hexylresorcinol (S.T. 37)	161
Mammalian Ringer's	168†
Mammalian Ringer's	288†
Mammalian Ringer's	120†
Mammalian Ringer's with trace of glucose	576

SEC. B

MEDIA WITH AMINO NITROGEN SOURCES

Larvae

Medium	Survival (in Hours)
Tryptone (5 per cent) in distilled water	144
Tryptone (5 per cent) in mammalian Ringer's containing macerated infected rat liver	120
Mammalian Ringer's containing infected liver-digest	48
Tyrode over peptone-digest agar (2 per cent)	120
Modified Tyrode (disodium phosphate) over infected rat liver-digest in gelatin and agar	120
Tyrode over peptone-digest agar (2 per cent)	146
Tyrode over tryptone-digest agar (1 per cent) plus trace of sodium taurocholate	100
Tyrode over tryptone-digest agar (1 per cent) plus trace of sodium taurocholate	169
Tyrode over tryptone-digest agar (1 per cent) plus trace of sodium taurocholate	211
Tyrode over tryptone-digest agar (1 per cent) plus trace of sodium taurocholate	48
Tyrode over peptone-digest agar (2 per cent) plus trace of acriflavine	48

Adults

Tyrode over tryptone-digest agar (1 per cent) plus trace of sodium taurocholate	77

* Killed by overheating. † It was not noted when these died.

RESULTS

I. ATTEMPTS AT *in vitro* CULTURE

1. *Culture experiments utilizing media without amino nitrogen sources.*—Survival in hours of larvae of *T. taeniaeformis* in media which did not contain amino nitrogen is listed in Section A of Table 1. These worms were placed in fresh media every 18 hours, on the average, for most of the experiments. The average initial number of larvae used, with the exception of the series in mammalian Ringer's solution, which survived 576 hours, was 5. In the case of the exception, 36 larvae were placed in 150 cc. of sterile mammalian Ringer's. The initial change to fresh medium was 13 days later. On the

fifteenth day the larvae were again changed to fresh Ringer's to which a few drops of sterile 20 per cent glucose solution was added. This same modification was made at subsequent medium changes on the twenty-first, twenty-second, and twenty-fourth days, respectively. These larvae were well-developed worms 5 months of age when removed from the rat, and they were maintained a minimum of 24 days (576 hours) *in vitro*. There was no sign of decomposition among the worms left in the flask at 37.5° C. upon subsequent examination several weeks later.

During the course of these culture experiments the effect of the larvae upon the pH of the medium was studied. A typical record is given in Table 2. Tyrode's solution, modified by a double portion of sodium bicarbonate, was used. The average elapsed time between successive pH readings was a little more than 15 hours. The average pH drop was 0.25 for the media in the container with 1 worm. For the container with 3 worms the average pH drop was 1.34. This change in the pH of the solution containing 3 worms was more than three times as great as the corresponding change with the single worm and may be due in part to the fact that movement of 1 worm probably stimulated others present in the same flask to move, the greater activity resulting in greater physiological changes. There was no evidence of bacterial development until the fourteenth day of the experiment, when Container II, with the 3 worms, appeared to have some precipitate present, and a slight cloudiness was observed. Two of the worms in this container were dead 25 hours later and were removed. The third was still alive 16 days after the start of the experiment.

Using sterile Tyrode's solution, modified by the substitution of disodium phosphate for the usual sodium bicarbonate, another series of pH readings was made. The initial pH for this solution was 7.6; and two flasks, each containing 3 larvae, were set up. The average drop in pH was 1.34 and 1.0, respectively, for an average interval of 16.1 hours. An-

TABLE 2

A COMPARISON OF CHANGES IN THE pH OF TYRODE'S SOLUTION CONTAINING *T. taeniaeformis* LARVAE

Medium: modified Tyrode's solution (double sodium bicarbonate). Amount: 20 cc.

Elapsed Time (in Hours) between Solution Changes	Initial pH	Container I (1 Worm)		Container II (3 Worms)	
		pH of Old Media	Drop in pH	pH of Old Media	Drop in pH
24	8.8	.....	.....	below	2.0
41	8.8	.....	.....	6.8	2.0
9	8.8	.....	.....	.....	.....
15	8.8	.....	.....	.....	.....
9	8.8	.....	.....	7.4	1.4
18	8.8	8.3	0.5	6.7	2.1
26.5	9.0	8.0	1.0	6.5	2.5
24	9.0	8.4	0.6	6.9	2.1
8.5	8.9	.....	.....	8.0	0.9
11.5	8.6	8.3	0.3	7.4	1.2
6.5	8.8	8.8	0.0	7.9	0.9
17	8.9	8.7	0.2	7.1	1.7
12	8.8	8.7	0.1	7.4	1.4
17	8.9	8.9	0.0	7.0	1.9
7.5	8.8	8.7	0.1	7.7	1.1
16	9.0	9.0	0.0	7.7	1.3
7.5	9.3	9.3	0.0	9.2	0.1
13.5	8.8	8.5	0.3	7.9	0.9
27.5	8.6	8.4	0.2	6.8	1.8
19	8.7	dead	dead	6.8	1.9
8.5	8.6	.....	.....	7.1	1.5
16	8.4	.....	.....	7.2*	1.2
8	8.4	.....	.....	7.6	0.8
45	8.6	.....	.....	6.7	dead

* Two of the 3 worms in Container II were dead at this time.

other series, using modified Tyrode's solution containing 0.01 per cent urea, produced an average drop in pH of 2.9 for an average period of 9.4 hours when 4 worms were present.

Five worms from an infected rat were placed in 20 cc. of sterile Ringer's solution, and five drops of hexylresorcinol (S.T. 37) were added to reduce surface

tension. Similarly, 4 other larvae from the same rat were placed in 20 cc. of autoclaved Ringer's solution for a control. The worms were changed to fresh, sterile media three times in the course of this experiment. Movement of worms was discerned in both flasks almost 7 days after the start of the experiment. No effect on vitality of the worms in a medium of reduced surface tension was apparent.

2. *Culture experiments utilizing media containing amino nitrogen sources.*—Survival in hours of larvae and adults of *T. taeniaeformis* in media which contained amino nitrogen sources is listed in Section B of Table 1. The average initial number of larvae used in each experiment was 4. In most of the experiments the worms were transferred to fresh, sterile media daily. Comparison of Sections A and B in Table 1 will show that survival was longer in media without amino nitrogen sources.

One group of 5 worms was placed in a medium formed of pancreatin-digested "immature" larvae of *T. taeniaeformis* which had been killed by heating to 45° C. Medium for a second group of 5 worms was composed of equal amounts of this digest and of mammalian Ringer's solution. The first medium seemed to be toxic immediately, but the larvae in the second medium lived a minimum of 48 hours.

An experiment was set up with non-autoclaved media. Twelve or more large larvae were added to each of three flasks, one of which contained 20 cc. of Tyrode's solution, while a second contained an equivalent of this same stock Tyrode over plain agar (2 per cent) and the third contained another volume of the Tyrode over a peptone digest made up in 2 per cent agar. One drop of toluol was added to each of the flasks. No movement was discernible even on the second day; and when it was established that the worms were dead, the experiment was terminated. It seems probable that the toluol was toxic even in this amount, which is approximately a dilution of 400 to 1.

A cat was sacrificed, and 33 worms were taken from the intestine. These adult *T. taeniaeformis* did not respond well to the Tyrode's solution over the tryptone-digest agar containing a trace of bile salt. These worms were changed to fresh media daily. Only 1 worm of the large number used showed motility at the end of 77 hours.

TABLE 3

A COMPARISON OF VITALITY OF LARVAE OF *T. taeniaeformis* IN MODIFIED TYRODE'S SOLUTION (NO GLUCOSE) WITH AND WITHOUT UNHEATED CAT SERUM

Medium	No. of Worms	Survival (in Hours) as Indicated by Motility
Tyrode with cat serum.....	2	24
Tyrode without serum.....	2	73
Tyrode with cat serum.....	1	8
Tyrode without serum.....	1	48
Mammalian Ringer's solution.....	1	96

One attempt at aerobic culture using cat serum was made. Five test tubes were set up. To each of four tubes, 10 cc. of modified Tyrode (no dextrose) was added; and to two of these four, an additional 1 cc. of cat serum was added. Ten cubic centimeters of mammalian Ringer's solution was used in the fifth tube. One or 2 larvae were placed in each of the tubes. The results are recorded in Table 3.

3. *Culture experiments with perfusion apparatus.*—A gravitational perfusion apparatus was constructed. A reservoir was placed above the incubator. Glass tubing led into the incubator to connect with a minimum of 6 feet of coiled glass

tubing in the top of the incubator. The medium was carried next into the culture flask (Erlenmeyer), and the flow was regulated so that the fluid dropped on the surface of the shallow solution containing the larvae of *T. taeniaeformis*. By regulating the height from which the fresh medium dropped and the depth of the medium containing the worms a certain amount of disturbance could be maintained in the latter solution. When several worms were present in a small flask, they had a tendency to knot and coil into a compact mass. This was largely prevented by placing sterile glass beads over the bottom of the culture flask. The overflow tube was a siphon which could be regulated so that the depth of the medium in the culture flask was controlled. Later, a modification of the "single-flask" perfusion setup was constructed so that three culture flasks were used in series. This was done because it was considered that some conditioning of the medium by the previous presence of worms might make it more favorable for *in vitro* maintenance. Table 4 gives the results of perfusion experiments with the previously discussed "occasional-change" method as a control.

4. *Anaerobic culture experiments.*—A number of experiments was tried utilizing modified-anaerobic and anaerobic culture methods. Four test tubes were set up, each containing 10 cc. of mammalian Ringer's solution containing 1 per cent glucose. The first tube was left as a control; but traces of urea, allantoin, and uric acid were added to the second, third, and fourth tubes, respectively. After one worm had been placed in each tube, Nujol was added over the Ringer's, so that the test tube was nearly filled. Then rubber stoppers were inserted tightly in these four tubes. It was considered that the worm would be in a medium which was nearly anaerobic after it had utilized oxygen already present. Later experiments on oxygen consumption indicate that the oxygen would be exhausted in

TABLE 4

A COMPILATION OF THE RESULTS OF PERFUSION EXPERIMENTS WITH TAPEWORM LARVAE COMPARED WITH THE METHOD OF OCCASIONAL CHANGE OF MEDIUM

Medium	Method	Survival (in Hours)
One bouillon cube per liter of 1 per cent glucose–mammalian Ringer's with a trace of sodium taurocholate	Single-flask perfusion	29
1 per cent glucose–mammalian Ringer's	Occasional change	72
	Single-flask perfusion	99
	Series-flask perfusion, flask (A)	71
	flask (B)	36
	flask (C)	56
Tyrode without glucose	Occasional change	113
1 per cent glucose–mammalian Ringer's	Single-flask perfusion	107
1 per cent glucose–mammalian Ringer's	Single-flask perfusion	78
	Series-flask perfusion, flask (A)	58
	flask (B)	51
	flask (C)	30

about 2 hours. A second set of tubes, using similar media, was set up; but these controls were cotton plugged and not covered with Nujol. Five days after the beginning of this experiment all worms showed motility; but the aerobic series appeared to be in better condition and, as a group, showed greater activity than those of the anaerobic series. All worms were changed to fresh media at this time, but newly autoclaved Ringer's was used with the anaerobic series in an attempt to keep the oxygen content minimal. Only the worms in glucose and glucose-urea solutions were alive on the sixth day.

A bacteriological method for maintenance of anaerobic conditions was utilized. Freshly ground tissues from a half-grown kitten which was not infected with adult *T. taeniaeformis* were placed in the lower half of test tubes. Various combinations of tissues were set up, both in an alkaline series with trypsin and in an acid series with pepsin, as follows:

GROUP A (ALKALINE SERIES)

(Trypsin and sodium bicarbonate; pH 7–9)

I. Intestine-muscle infusion
II. Liver-muscle infusion
III. Muscle infusion
IV. Stomach-spleen-muscle infusion

GROUP B (ACID SERIES)

(Pepsin and HCl; pH 1–4)

V. Stomach-spleen-muscle infusion
VI. Muscle infusion
VII. Liver-muscle infusion
VIII. Intestine-muscle infusion

The material in each tube was covered with mammalian Ringer's solution. One to 3 worms removed from their cysts were placed in each test tube, except in the case of tube No. VI, in which 3 larvae still in their cysts were placed. One larva in its cyst was also placed in test tubes Nos. I and II. A trace of bile salt (sodium taurocholate) was added to each tube of the alkaline series. Movement of worms could not be discerned in any of the test tubes after the first few hours. None of the worms were living on the sixth day, when the larvae were removed from the anaerobic tubes and carefully examined. However, the worms of the acid series, both when removed from cysts and within the cysts, were in better condition than were those of the alkaline series.

The anaerobic medium of Brewer (1940) was utilized in certain experiments. This contains pork infusion solids, peptone, and glucose as food sources and was buffered at a pH of 7.5. Six-inch test tubes, which were $\frac{3}{4}$ inch in diameter, were utilized; and these were filled to a height of 7 cm. with about 15 cc. of broth. Five test tubes were set up, and 2–4 small larvae were placed in each tube. Motility in the worms was observed after 27 hours in this medium, although it was reduced under these anaerobic conditions. Since much agitation of the test tube increased temporarily the amount of oxygen in the broth, attempts to induce movement of the worms in anaerobic series was restricted to a gentle shaking of the containers.

Later this experiment was repeated. From 2 to 4 worms were placed in each of four test tubes, while in the fifth, two cysts containing worms were used. Motility was observed after about 5 hours, but subsequent observation failed to note worm activity. Two of the tubes in this series developed a local precipitate suspended around the parasites. This probably indicated anaerobic bacterial growth. Although the worms were dead on the fifth day, when they were removed from the test tubes and examined, they all appeared to be in good condition.

This technique was modified somewhat as follows: 2 small worms were

placed in a test tube which contained a shorter column of anaerobic broth, since, if these parasites possessed a facultative metabolism, an incompletely anaerobic environment might be more beneficial. About 2 cc. of chick embryonic extract were added to this medium. Three days later, when there was no indication of life, these larvae were transferred to a cotton-plugged Erlenmeyer flask containing mammalian Ringer's solution. The methylene blue which they had derived from the anaerobic medium turned the worms blue upon exposure to the oxygen of the air.

II. RESPIRATION STUDIES

After several series of preliminary experiments to develop technique, a series of determinations was made on the rate of oxygen consumption by larvae of *T. taeniaeformis*. A rat was killed, and 11 worms were removed from the liver. Seven of this group were placed in the incubator in mammalian Ringer's (no glucose). After about 2 hours, to allow for acclimatization of the larvae, they were transferred to the respiratory chamber in a fresh volume of the mammalian Ringer's stock solution in which the oxygen content was in approximate equilibrium with the atmosphere. A 2-cc. sample was tested immediately for oxygen content. Thereafter, at 10-minute intervals fresh samples from the respiratory chamber were tested similarly for oxygen content. In like manner, the effect on oxygen consumption of various concentrations of potassium cyanide (Table 5) made up in mammalian Ringer's solution was measured and compared with the rate in normal Ringer's. In every case oxygen consumption of these larvae was measured in the normal solution immediately prior to the tests with each cyanide solution, respectively. Table 6 contains the observed and calculated results of the initial series, which measured oxygen consumption of these 7 larvae. It was found that the observed data fitted a straight line. Accordingly, the straight-line formula ($y = mx + b$) was used to arrive at the calculated results. The mean oxygen consumption both for the observed and for the calculated data is listed in Table 7. Larval Lot A represents the 7 larvae just referred to above,

TABLE 5

CYANIDE CONCENTRATIONS IN MAMMALIAN RINGER'S SOLUTION (WITHOUT GLUCOSE) USED IN RESPIRATION STUDIES OF A GROUP OF 7 *T. taeniaeformis* LARVAE AT VARYING PERIODS AFTER REMOVAL FROM CYSTS IN THE RAT HOST

Cyanide Concentration	Time (in Hours) after Removal from Cysts	Cyanide Concentration	Time (in Hours) after Removal from Cysts
0.0002 N....	5	0.002 N....	61
0.0001 N....	22	0.0015 N....	91
0.005 N....	38	0.001 N....	159

TABLE 6

SHOWING THE RESULTS OF MEASUREMENTS OF OXYGEN CONSUMPTION OF *T. taeniaeformis* LARVAE IN MAMMALIAN RINGER'S SOLUTION, TAKEN ABOUT 2 HOURS AFTER REMOVAL FROM THE RAT*

Formula: $y = mx + b$; $m = 0.053$; $b = 0.57$; $y = (a - x)$.

Elapsed Time (in Minutes)	Amount of Sodium Thiosulphate Used (Cc.)	Oxygen Equivalent (Cc. per Liter)	Actual Oxygen Uptake ($a - x$)	Calculated Oxygen Uptake
Initial = a...	2.0	5.6	.....	.....
10.....	1.6	4.48	1.12	1.10
20.....	1.4	3.92	1.68	1.63
30.....	1.3	3.64	1.96	2.16
40.....	1.0	2.80	2.80	2.69
50.....	0.85	2.38	3.22	3.22

* These larvae weighed 1.5 gm., approximately.

whereas larval Lot B represents a group of 24 smaller larvae whose total weight was only slightly more than one-third that of the 7 larvae and which was used as a check for the previous comparisons. With similar cyanide concentrations results are comparable for the two groups of larvae.

Data for the rates of oxygen consumption at 36 and at 60 hours are expressed in curves (Fig. 1) where the amount of oxygen consumption is plotted against the time interval. Curves were calculated by the "method of least squares," using the formula $y = mx + b$; and the observed points are superimposed on the calculated curves. The 0.005 N and the 0.002 N potassium cyanide–Ringer's used, respectively, at this phase of the series had the most pronounced effect on lowering respiration rate of any of the concentrations utilized. The 0.0002 N and the 0.0001 N potassium cyanide-Ringer's had little or no effect, apparently, upon the rate of respiration, as compared with the corresponding determinations in normal Ringer's; but the cyanide concentration (0.005 N) used at 36 hours seems to be affecting the rate of respiration of these larvae in normal Ringer's solution 24 hours later (i.e., at 60 hours), as shown by its lesser slope, when compared with respiration in normal Ringer's at the 36-hour period. This same delayed recovery from the effect of cyanide on respiration is apparent in subsequent studies. It is evident that some recovery from the effect of cyanide is occurring; but under the conditions of the experiment, time sufficient for complete recovery had not been allowed.

Figure 2 portrays graphically the

TABLE 7

SUMMARY OF RATES OF OXYGEN CONSUMPTION OF TWO LOTS OF *T. taeniaeformis* LARVAE IN MAMMALIAN RINGER'S SOLUTION WITH AND WITHOUT KCN

Larval Lot	Series	Initial Oxygen Content (Cc. per Liter)	Cyanide Strength	Elapsed Time				Mean Amount of Oxygen Used for Selected Number of Larvae (in Cc. per Liter) (Measurements Taken at 10-Minute Intervals)	
				Total Hours *in vitro*	Series in Normal Ringer's (in Minutes)	Series in Cyanide Ringer's (in Minutes)	Hours since Last Cyanide Treatment	Observed	Calculated
A.....	1	5.6	.....	.2	50	.....	.....	2.16	2.16
	2	6.02	0.0002 N	.5	.....	50	.....	2.38	2.38
	3	5.44	.....	20	90	.....	14	2.48	2.54
	4	5.6	0.0001 N	22	.....	90	16	2.28	2.23
	5	5.88	.....	36	50	.....	13	2.87	2.67
	6	5.6	0.005 N	38	.....	70	15	1.58	1.58
	7	4.76	.....	60	50	.....	21	1.89	2.03
	8	5.04	0.002 N	61	.....	70	22	1.22	1.22
	9	4.71	.....	90	60	.....	28	1.67	1.72
	10	5.46	0.0015 N	91	.....	80	29	1.66	1.67
	11	5.04	.....	157	80	.....	64	2.45	2.49
	12	5.25	0.001 N	159	.....	70	66	1.47	1.45
	13	6.44	.....	288	70	.....	130	2.32	2.32
	14	5.04	.....	364	80	.....	207	1.82	1.82
B.....	1	4.81	.....	48	60	.....	.....	1.64	1.66
	2	5.12	0.001 N	50	.....	60	.....	1.38	1.38
	3	5.6	.....	72	60	.....	21	2.12	2.13
	4	5.6	0.001 N	74	.....	60	23	1.70	1.70

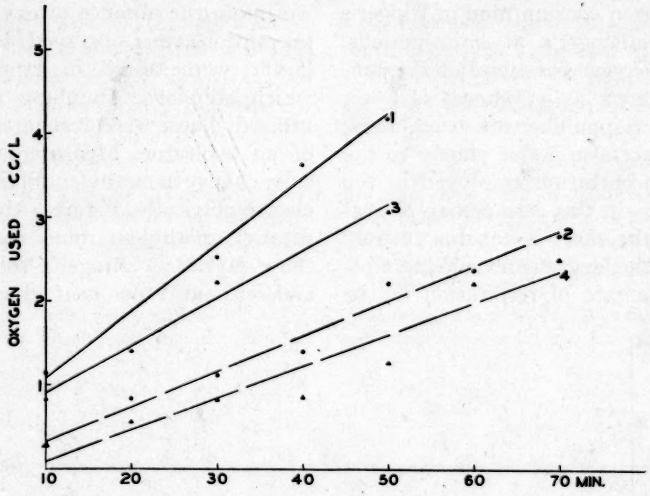

FIG. 1.—Showing the calculated and observed rates of oxygen consumption of 7 larvae of *T. taeniaeformis* approximately 36 and 60 hours after removal from the host. (1) Ringer's (36 hours), solid circle. (2) 0.005 N. KCN-Ringer's (36 hours), open circle. (3) Ringer's (60 hours), solid triangle. (4) 0.002 N. KCN-Ringer's (60 hours), open triangle.

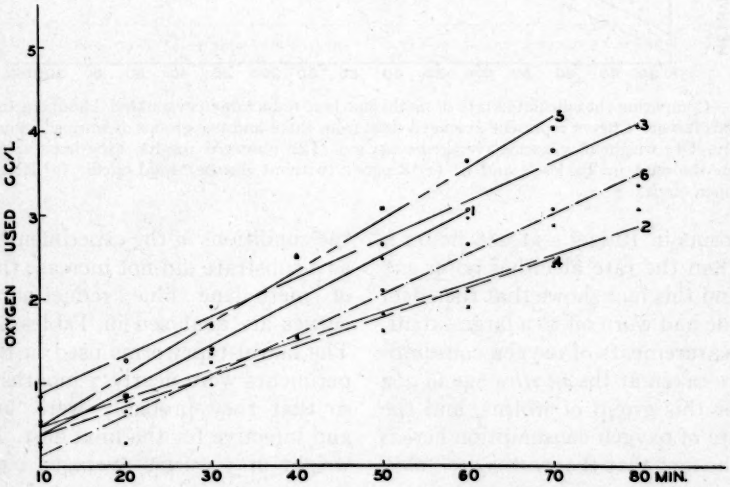

FIG. 2.—Showing the calculated and observed rates of oxygen consumption of the same 7 larvae of Fig. 1 at later periods after removal from the host. (1) Ringer s (90 hours), solid circle. (2) 0.0015 N. KCN-Ringer's (90 hours), solid triangle. (3) Ringer's (157 hours), open circle. (4) 0.001 N. KCN-Ringer's (157 hours), cross. (5) Ringer's (288 hours), open square. (6) Ringer's (364 hours), solid square.

rates of oxygen consumption in Ringer's and cyanide-Ringer's at later periods. The rate of oxygen consumption in mammalian Ringer's at 157 hours is lower than the corresponding rate at 90 hours; but this effect also is due chiefly to the cyanide concentration employed in the earlier series, in this case 0.0015 N cyanide. That the effect is not due entirely to "age" of the larvae is revealed by noting that the rate of respiration of the worms in the absence of oxygen. Sterile precautions were observed. From 1 to 5 larvae were tested in experiments in which standard Thunberg tubes were utilized. These were evacuated by means of an aspirator. Measurements of the color change in methylene blue were taken electrometrically. Figure 3 compares the rate of methylene blue reduction by these larvae in Ringer's solution with and without 1 per cent glucose. Under

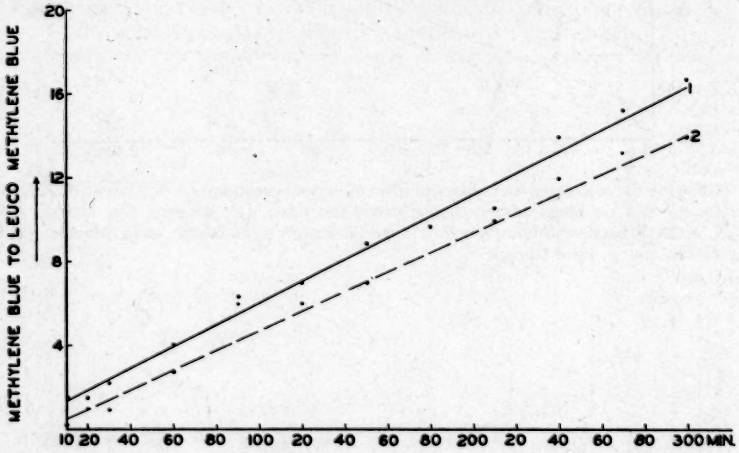

FIG. 3.—Comparing the calculated rate of methylene blue reduction in evacuated Thunberg tubes by *T. taeniaeformis* larvae. Curves represent averaged data from three and two groups of worms (5 worms each), respectively. Dry weight of 5 worms averaged 0.045 gm. The observed results, experimental points, are taken from the data in Tables 8 and 9. (1) Ringer's (without glucose), solid circle. (2) Ringer's (with glucose), open circle.

same worms in Ringer's at 288 hours is higher than the rate at either 90 or 157 hours, and this fact shows that the effect of cyanide had worn off to a large extent. Final measurements of oxygen consumption were taken at the *in vitro* age of 364 hours for this group of worms, and the lower rate of oxygen consumption here is due to the fact that the worms are "old" from the standpoint of *in vitro* culture.

A series of experiments on fresh, whole larvae of *T. taeniaeformis* was conducted to determine whether methylene blue is reduced to leuco methylene blue by these the conditions of the experiment, glucose as a substrate did not increase the speed of methylene blue reduction. These curves are analyzed in Tables 8 and 9. The larval tapeworms used in these experiments were nearly 7 months of age, so that they probably were "mature" and infective for the final host. The dry weight of 5 worms averaged 0.045 gm. One group of larvae, approximately 3 months of age and whose dry weight for 5 worms averaged 0.014 gm., were studied by means of the Thunberg technique, using mammalian Ringer's solu-

tion without glucose. Methylene blue reduction by these worms is plotted in Figure 4, and the curves all reveal an early lag. It seems probable that there is a dehydrogenase present which is causing methylene blue reduction and that it is acting upon some substrate other than glucose. After the completion of the ex-

TABLE 8

SHOWING THE RATE OF METHYLENE BLUE REDUCTION IN EVACUATED THUNBERG TUBES OF *T. taeniaeformis* LARVAE IN MAMMALIAN RINGER'S SOLUTION CONTAINING NO GLUCOSE*

Formula: $y = mx + b$; $m = 0.052$; $b = 0.80$.

Elapsed Time in Minutes (x)	Observed Change in Methylene Blue (y)	Calculated Change in Methylene Blue (y Calculated)
10	1.5	1.32
20	1.5	1.84
30	2.25	2.36
60	4.17	3.92
90	6.33	5.48
120	7.0	7.04
150	8.8	8.60
180	9.7	10.16
210	10.5	11.72
240	14.0	13.28
270	15.3	14.84
300	16.8	16.40

* These data are the averaged results of three series utilizing 5 larvae each.

TABLE 9

SHOWING THE RATE OF METHYLENE BLUE REDUCTION IN EVACUATED THUNBERG TUBES OF *T. taeniaeformis* LARVAE IN MAMMALIAN RINGER'S SOLUTION CONTAINING 1 PER CENT GLUCOSE*

Formula: $y = mx + b$; $m = 0.047$; $b = 0.04$.

Elapsed Time in Minutes (x)	Observed Change in Methylene Blue (y)	Calculated Change in Methylene Blue (y Calculated)
10	0.25	0.51
20	1.0	0.98
30	1.0	1.45
60	2.75	2.86
90	6.0	4.27
120	6.0	5.68
150	7.0	7.09
180	7.0	8.50
210	10.0	9.91
240	12.0	11.32
270	13.25	12.73
300	14.0	14.14

* These data are the averaged results of two series utilizing 5 larvae each.

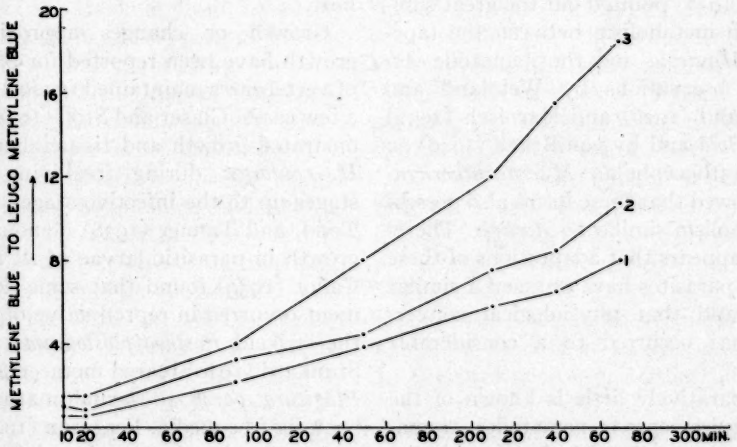

FIG. 4.—Showing rate of methylene blue reduction in a group of larvae of *T. taeniaeformis* approximately 3 months of age. Dry weight for 5 worms averaged 0.014 gm. (1) Average of two series (2 worms each). (2) Average of 2 series (2 worms each). (3) One series (3 worms).

periments with evacuated Thunberg tubes, the larvae were found to be active and in good condition when returned to aerobic media.

DISCUSSION

It is recognized that more anatomical differences than similarities exist between such diverse groups as Acanthocephala, nematodes, and flatworms. Among this last group the tapeworms are distinguished from the trematodes by the absence of a gut in the former—a feature which at once suggests profound differences. Absence of an intestine determines the location for adult tapeworms; and, with the exception of forms that occur in bile and hepatic ducts, they are normally found in the host intestine. There is, however, a major feature that these diverse groups have in common, and it is particularly true when these forms are parasitic in the same or in closely related hosts. Each group of these parasites has undergone precise adaptation to a new and relatively similar environment, and this environment is very complex and rigorously controlled. Von Brand (1938) pointed out the great similarity in metabolism between the tapeworm *Moniezia* and the nematode *Ascaris*. Observations by Weinland and von Brand (1926) and Harnisch (1932) on *Fasciola* and by von Brand (1940) on the acanthocephalan *Macracanthorhynchus* showed that these forms also possess a metabolism similar to *Ascaris*. Therefore, it appears that adaptations of these various parasites have pursued a similar course and that physiological convergence has occurred to a considerable degree.

Comparatively little is known of the food requirements of nematodes, trematodes, and cestodes. McCoy (1929) and Lapage (1933) reared various nematode larvae on pure cultures of bacteria. Ackert and co-workers (1935, 1936) have shown that *Ascaridia lineata*, a nematode parasite of chickens, takes nourishment from food in the intestinal lumen of the host. Glaser and Stoll (1938) reared the free-living stages of the sheep-stomach worm *Haemonchus contortus* in sterile media. Von Brand and Simpson (1944) maintained *Eustrongylides* larvae *in vitro* for over 2 years. Weinland and von Brand (1926) agreed with an earlier worker (Müller, 1923) that *Fasciola hepatica* feeds on tissue elements and inflammatory exudates rather than on blood. Wardle (1937*a*) showed that about 32 per cent of the dry weight of *Moniezia* is polysaccharide, and he stated that "glucose seems definitely the one carbohydrate requirement of tapeworms." Salisbury and Anderson (1939) found 30 per cent of the dry weight of *T. taeniaeformis* larvae to be glycogen. Chandler (1943) showed that the tapeworm *Hymenolepis diminuta* was dependent upon carbohydrate, but not on protein, in the diet of the host. He suggested that tapeworms obtain some nutrient material from direct contact with tissues of the host.

Growth or changes approximating growth have been reported for parasites of vertebrates maintained *in vitro* in only a few cases. Glaser and Stoll (1938) demonstrated growth and tissue changes in *H. contortus* during free-living larval stages up to the infective stage. Ackert, Todd, and Tanner (1938) demonstrated growth in parasitic larvae of *A. lineata*. Callot (1939) found that some development occurred in reproductive organs of the strigeid *Posthodiplostomum cuticola*. Stunkard (1940) reared metacercariae of *Plagitura parva* to sexual maturity in bacteria-free media. Ferguson (1940) got egg production *in vitro* in adults of *P. minimum* which had been reared from metacercariae. Von Brand and Simpson

(1944) found one adult male of *Eustrongylides ignotus* which had molted *in vitro* from the larval stage.

Hydrogen-ion concentration of media has been studied by various authors. Stunkard (1932) found a pH of 7.3 optimum for *Crepidobothrium lönnbergi* in cultures. McCoy (1935) considered that wide variations in pH, as well as factors of temperature and oxygen tension, were not harmful to parasites generally. Hoeppli, Feng, and Chu (1938) conclude that a pH of 7.3–7.6 is generally favorable; but a pH of 6.93 and of 6.5 were used respectively by Ackert, Todd, and Tanner (1938) with *Ascaridia* and by Ferguson (1940) studying *P. minimum* of birds. Von Brand (1943) found that oxygen consumption of *Eustrongylides* larvae did not vary appreciably over a pH range of 3.4–8.3, but von Brand and Simpson (1944) found that larvae of this species survived longest when the pH of the medium was near the neutral point. In the present study, *T. taeniaeformis* larvae have survived a pH as high as 9.3 and as low as 4.6. Furthermore, these worms have withstood gradual change in the pH of the medium from 8.8 to 6.5 and abrupt change when returned to the higher pH with no chance for gradual acclimatization. These larvae are tissue parasites; but the ability (some survived the treatment for as long as 16 days) to withstand considerable change in pH may indicate that the worms studied had reached an age at which they would be infective in the intestine of the final host, where they might be subjected to considerable variation in pH. For adult worms already established in the intestine, tolerance *in vitro* for a slightly greater pH range has been reported by Cook and Sharman (1930) for the tapeworm *Moniezia* and by Davey (1937) for sheep nematodes.

In some preliminary experiments the incubator temperature fluctuated greatly; yet this factor did not seem to be particularly important. Later a defective switch, in an electric incubator, allowed the temperature to drop to that of the room. Worms present in the incubator at this time did not seem to be adversely affected by this lower temperature when restored to normal body temperature. It is well known that such helminths as the microfilaria live much longer at ice-chest temperatures than at room temperature. Cook and Sharman (1930) found the metabolic rate of tapeworms lower at 20° C. than at 37° C., but the worms resumed the rate at body temperature when returned to it. In the present experiments, in two cases, rats found dead by the animal-room attendant and not immediately reported were placed in the cold room (14° C.), where they were left at least 24 hours before being examined. Live *T. taeniaeformis* larvae were recovered and were maintained for 8 and 12 days, respectively, in the incubator at 37.5° C. McCoy (1935) stated that parasites are relatively insensitive to wide variations in certain environmental factors such as pH, oxygen tension, and temperature; and this statement holds for the larvae of *T. taeniaeformis*.

Surface tension as a physical factor affecting the *in vitro* survival of parasitic worms has not previously been studied. Davey (1938) correlated bile salt concentration with the distance from the bile-duct opening at which various sheep nematodes normally occurred in the intestine. De Waele (1934) and Edgar (1941) used bile salts to stimulate evagination of scolices of larval tapeworms. In the present study, neither bile salts nor hexylresorcinol was more efficient than the controls in maintaining larvae *in vitro*.

The question of oxygen tension has not been answered. According to all re-

ports, the presence of oxygen in a medium is not harmful to the worms. In most cases they utilize such oxygen. Friedheim (1934) found that the eggs, plerocercoids, and adults of *Diphyllobothrium latum*, the broad tapeworm, absorbed oxygen; and Harnisch (1933) found with *Triaenophorus nodulosus*, a tapeworm of fish, that absorption was proportional to the oxygen pressure of the environment, a condition that holds for the larvae of *T. taeniaeformis* for oxygen concentrations of salt solutions in equilibrium with the atmosphere.

On the other hand, parasites living in the intestine would have to utilize oxygen at a very low tension, or possess an anaerobic metabolism, or both, since little free oxygen is present in this organ (von Brand, 1938). Weinland and von Brand (1926) and Harnisch (1932) showed that certain fatty acids were produced in the anaerobic metabolism of the fluke *F. hepatica*. Von Brand (1929, 1933) and Alt and Tischer (1931) showed that fatty acids and lactic acid make up the principal fermentative products of the tapeworm *Moniezia expansa*. Von Brand (1937) measured anaerobic glycogen consumption in *Ascaris* and came to the conclusion that respiration in this nematode is mainly anaerobic. However, Davey (1938) showed that in complete absence of oxygen various sheep nematodes died within 48 hours; and in present experiments *T. taeniaeformis* larvae did not survive so long under anaerobic (5 days maximum) as under aerobic (24 days) conditions, nor were they so active. Slater (1925) found that *Ascaris*, periodically stimulated to activity, did not survive so long in an anaerobic environment as when oxygen was available; and he believed that this nematode went into a state of "suspended animation" when deprived of oxygen. It is possible that this explanation fits *T. taeniaeformis* larvae, but in the larval stage these worms are tissue parasites and presumably would have to undergo considerable adaptation when taking up their abode in the cat intestine as adults. There is little to indicate whether this adaptation is a gradual or a saltatory process stimulated by digestive juices or other factors of the cat intestine.

Mendelsohn (1935) maintained *T. taeniaeformis* larvae over a month, utilizing the roller-tube method of tissue culture. These larvae, however, were only 15 days old and approximately 1 mm. in length. Oxygen diffusion would presumably not be a problem for larvae of this size. Using a similar argument, von Brand (1938) reconciled the aerobic metabolism of the sheep nematode *Ostertagia* with the anaerobic metabolism of *Ascaris*. He believed that, since *Ostertagia* was an incomparably smaller organism than *Ascaris*, tissues would be thinner and body surface relatively much larger, so that oxygen diffusion, even at low tensions, conceivably might be the answer to the aerobic respiration of the sheep nematode.

Larvae of *T. taeniaeformis* are not infective for the cat host until after a 7-month sojourn in the rat host, so that *in vitro* culture of worms approaching "larval maturity" is a very different problem from that of young, immature stages. In the present experiments 5-month-old larvae of *T. taeniaeformis* were maintained aerobically in a sterile medium containing only a trace of glucose, and these larvae showed motility 24 days after the beginning of the experiment.

The respiration of the larvae of *T. taeniaeformis* has been found to be cyanide-sensitive in part, and this cyanide effect has been shown to be reversible. Friedheim (1934) showed that the respiration of eggs, plerocercoid larvae, and adults of *D. latum* possessed a respiration

which was sensitive to cyanide. The eggs were almost completely cyanide-sensitive, whereas the adult was only partly so. Friedheim also reported that the adult worm showed two absorption bands of cytochrome C. It is probable that cytochrome is responsible for the cyanide-sensitive respiration of the larvae of *T. taeniaeformis*, since cyanide is a specific poison for this phase of respiration in aerobic animals as well as in plants. Meldrum (1934) stated that cytochrome "can almost be stated to be a component of every aerobic cell, and conversely that it is absent—or almost absent—from cells which live anaerobically." Stannard, McCoy, and Latchford (1938) found that oxygen consumption of *Trichinella spiralis* larvae was inhibited by cyanide.

Allee and Oesting (1934) showed that some aquatic animals produce sufficient nitrite to effect Winkler determinations of oxygen. Since the unmodified Winkler method was used in present studies, some error might be due to nitrite production by the tapeworm larvae. However, the consistent reduction in the rate of respiration by cyanide and the directly proportional ratios of varying cyanide concentrations to the decrease in rate of oxygen consumption indicate that the results are valid.

Experiments with *T. taeniaeformis* larvae in evacuated Thunberg tubes have shown that these worms were able to reduce methylene blue slowly. Since nearly one-third of the dry weight of these larvae is due to glycogen (Salisbury and Anderson, 1939) and since no appreciable difference occurred in methylene blue reduction in mammalian Ringer's with or without 1 per cent glucose, it may be that the dehydrogenase suggested by the methylene blue reduction is acting upon glycogen as a substrate. It is interesting to note that von Brand (1929, 1933) observed succinic acid in the medium after a study of the anaerobic decompositon of glycogen by *Moniezia*. Wardle (1937*a*), studying this same tapeworm, showed that glycogen content increased when the medium contained glucose up to 1 per cent but not when the glucose content exceeded this amount.

The addition of unheated cat serum made the medium less favorable for the survival of *T. taeniaeformis* larvae *in vitro*. Hoeppli, Feng, and Chu (1938) reported injection of cat serum, among other things, into cysts of larvae of this same species. Their results were less successful than with worms removed from cysts and maintained in similar media. It seems probable that pressure effects caused by the injection into the cyst may have affected the vitality of these larvae, but no mention was made of this feature.

In the present studies bacterial contamination had occurred in the media of certain series. Unquestionably, these bacteria influenced pH changes in the media, and they also may produce materials toxic to the worms. However, in one series of the present experiments, larval tapeworms survived longer in contaminated than in sterile media. Microflora normally in the intestine have not been shown to be harmful and may be beneficial to metazoan inhabitants of that organ.

Various digests of tryptone, peptone, uninfected rat liver, infected rat liver, and various tissues from an uninfected kitten have been utilized in aerobic and anaerobic conditions and with perfusion methods as well as with media in which excretory products of the experimental animals presumably were allowed to accumulate. The one conclusion that can be reached here is that the simpler media, in general, are more effective in maintaining larvae of *T. taeniaeformis in vitro* than are more complex media. Green and

Wardle (1941) found greatest longevity of *Hymenolepis in vitro* in a very dilute medium in Tyrode.

CONCLUSIONS

1. Well-developed larvae of *T. taeniaeformis*, after 5 months' development in the intermediate host, were maintained alive for 24 days. This constitutes the longest period that larvae which are approaching "maturity" have been kept alive.

2. A large number of media were used in attempts to find a successful basis for the *in vitro* culture of *T. taeniaeformis* larvae and adults. Survival of these larvae was longer in simple than in complex media.

3. It has been found that larvae of *T. taeniaeformis* survive longer under aerobic than under anaerobic conditions.

4. Evidence is presented that these larvae, which are tissue parasites in the rat, are able to withstand gradual pH changes from 9.3 to 4.6 and abrupt changes from 6.5 to 8.8.

5. It has been shown that these larvae, when placed in media of reduced surface tension, lived no longer than controls.

6. It was observed that larvae of *T. taeniaeformis* were able to survive temperature changes of considerable range (14°–37° C.).

7. It has been shown that cat serum (unheated) was less favorable for the survival of these larvae, removed from their cysts and maintained *in vitro*, than were the controls which contained no serum.

8. It has been found that oxygen consumption in *T. taeniaeformis* larvae is proportional to oxygen tension in aerobic respiration when the medium is in equilibrium with air.

9. In the course of studies on the metabolism of *T. taeniaeformis* evidence is presented for the first time that the respiration of larval stages of this tapeworm are cyanide-sensitive in part and that this cyanide inhibition is reversible.

10. New evidence is presented that the larvae of *T. taeniaeformis* are able to reduce methylene blue in a medium devoid of oxygen. The Thunberg technique was utilized. Preliminary experiments revealed no difference in methylene blue reduction with or without a glucose substrate.

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SOME FACTORS AFFECTING EXCYSTMENT IN THE CILIATE TILLINA MAGNA[1]

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IT IS generally agreed that the emergence of ciliated protozoa from their resting cysts is effected by certain environmental changes which stimulate the resting organisms to differentiate and escape from their cyst membranes. Considerable evidence indicates that the most effective excystment-inducing substances are of an organic nature. For example, Mast (1917) and Beers (1926) regularly activated the cysts of *Didinium nasutum* with timothy-hay infusion from *Paramecium* cultures, and Weyer (1930) reported that only the organic constituents of the medium were effective in inducing excystment in *Gastrostyla steinii*. By using bacterized gelatin solutions Weyer attempted to identify the active constituents with the end-products of protein decomposition by bacteria. Barker and Taylor (1933), in work on *Colpoda cucullus*, found that various extracts of plant and animal tissues, such as alfalfa infusion, yeast autolysate, and beef extract, were very effective; and their evidence from incineration experiments with yeast autolysate indicated again that only the organic constituents of such extracts were responsible for excystment. Thimann and Barker (1934), working likewise on *C. cucullus*, investigated the excysting capacity of a considerable number of amino and other organic acids but found them to be inactive. These findings, together with the observation that *sterile* timothy-hay infusion was highly active, led them to conclude that Weyer's hypothesis was untenable and prompted them to undertake the isolation of the active principles in hay infusion. They found that at least two substances—an ether-soluble and an ether-insoluble—were involved and that combinations of the two were more active than either alone. Haagen-Smit and Thimann (1938), continuing the analysis, found that the ether-soluble fraction contained a number of organic acids, among them succinic, fumaric, acetic, lactic, and oxalic. These acids, as well as citric, tartaric, and malic, in the form of their K or Na salts, proved to possess marked excysting properties; and the activity of the salts in admixture

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was found to exceed the sum of the activities of the individual components. This property was designated as a "summation effect." The ether-insoluble fraction proved to have a high carbohydrate content. While this fraction appeared to have little intrinsic excysting activity, it proved to be important as a cofactor, in that its presence produced a fourfold increase in the activity of salt mixtures. By using a solution of salts of organic acids which contained a sugar mixture as a cofactor, the authors reconstituted a "hay infusion" which had approximately 75 per cent of the activity of natural hay infusion. Thus the experiments provided convincing proof of the presence of specific excystment-inducing substances in plant infusions.

On the other hand, there is evidence that excystment is not dependent exclusively on the presence of organic compounds in the medium. Changes in hydrogen-ion concentration have been cited as important in inducing excystment (Koffman, 1924; Darby, 1929), though the preponderance of evidence indicates that such changes are without significant effect (Weyer, 1930; Barker and Taylor, 1933; Johnson and Evans, 1940). Garnjobst (1928, 1937) induced excystment in *Euplotes taylori* and *Stylonethes sterkii* merely by adding distilled water to the old encystment medium, a fact which indicates that changes in the physical properties of the medium may suffice to produce excystment. Furthermore, it is well known that the dried cysts of many ciliates will become active upon immersion in various plant infusions and extracts—e.g., *Uroleptus mobilis* (Calkins, 1919), *S. sterkii* (Garnjobst, 1937), and *C. cucullus* (Goodey, 1913; Bodine, 1923; Taylor and Strickland, 1935)—but it is not clearly established that such organic materials are absolutely necessary. In fact, Garnjobst was able to activate the dried cysts of *Stylonethes* with distilled water alone; and Goodey, referring presumably to dried as well as wet cysts, states that in *Colpoda* "excystation takes place quite freely in distilled water." Finally, Johnson and Evans (1940) were able to distinguish two types of resting cysts in *Woodruffia metabolica:* small, thick-walled "stable" cysts and large, thin-walled "unstable" cysts. The stable cysts require for their activation certain organic substances, whereas the unstable cysts do not require an organic medium but can be activated by various physical changes, such as a decrease in temperature, the replacement of the encystment medium with distilled water, or a change in the salt content of the medium.

From this review of a representative part of our knowledge of the factors that affect excystment, the need of further work is manifest. Hence, the present study was undertaken in an attempt to identify certain of the external changes, whether chemical or physical, which induce excystment in the holotrichous fresh-water ciliate *Tillina magna.*

THE CILIATE, ITS RESTING CYSTS, AND THE EXCYSTMENT PROCESS

Like *Woodruffia* and *Colpoda, Tillina* is a member of the Colpodidae and reproduces by division within a thin-walled temporary cyst. The general structure and method of division of *T. magna* were first described by Gruber (1879). Further contributions on structure, behavior, and reproduction were made by Gregory (1909), Ilowaisky (1921), Bresslau (1922), and Kahl (1931). While Ilowaisky calls his organism *Pseudocolpoda cochlearis cienkowskii,* it is unmistakably *T. magna,* as Kahl (p. 282) points out. The most thorough study of structure in a species of *Tillina* is that of Turner (1937, 1940), who describes

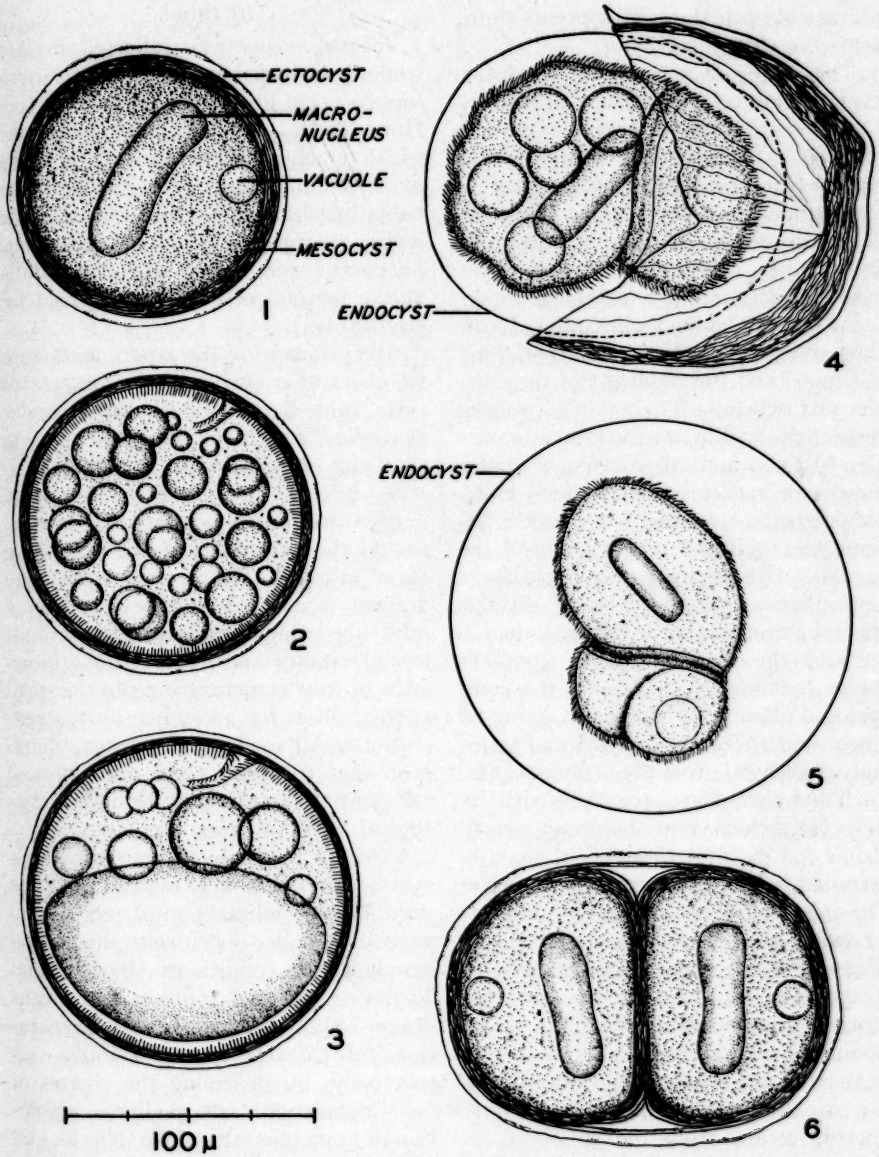
as a new species *T. canalifera*, which presumably differs from *T. magna* chiefly in the possession of a system of from seven to nine longitudinal canals which feed the contractile vacuole. These canals, Turner assumes, are absent in *T. magna*. Although such canals are unmentioned by Gruber, Gregory, or Ilowaisky, all of whom describe only a simple vacuole, one of Ilowaisky's figures shows clearly seven doubly contoured longitudinal markings which in number, size, and general arrangement agree with the canals of *T. canalifera;* and Kahl states somewhat emphatically that six longitudinal feeding canals are specifically diagnostic of *T. magna*. The specimens at my disposal show from six to ten canals, but the canals of the living organism readily elude observation in that they are difficult to distinguish optically and are frequently obscured by the food vacuoles. They may be seen to best advantage in living specimens which have just excysted, and therefore lack food inclusions, or in specimens stained with Delafield's hematoxylin, in which they appear as transparent tubules against a uniformly blue cytoplasmic background. Since these specimens agree with Kahl's diagnosis of *T. magna*, not only as regards the canals but also as regards size, shape of pharynx, presence of a dorsal protuberance around the contractile vacuole, and a diagonal buccal groove on the dorsal surface, I am compelled to regard them as *T. magna* and, further, to look on *T. canalifera* as a synonym of *T. magna*.

When subjected to certain unfavorable environmental conditions, *T. magna* produces both single and double resting cysts, as does *C. cucullus* (Barker and Taylor, 1931), though fully 80 per cent of the cysts observed in the present study were single ones. Single cysts (Fig. 1) are spherical, or nearly so, and in diameter vary from 90 μ to 120 μ; double cysts (Fig. 6) are oblately spherical and measure about 150 $\times$ 100 μ, with considerable variation.

Like the resting cyst of *Woodruffia*, the single cyst of *Tillina* has three membranes. The outermost or ectocyst (Fig. 1) is glutinous, transparent, and relatively thin and serves for attachment. Beneath it is the mesocyst ("endocyst" of some authors), a heavier, fibrous or laminated, slightly brownish membrane, which is the chief protective component of the cyst. Innermost is the endocyst ("intimocyst" of some authors), an extremely thin, transparent membrane, indistinguishable in the passive cyst but clearly visible in the late stages of excystment (Figs. 4 and 5).

The chief structural components of the protoplast are the finely granular cytoplasm; a single nonpulsatile, peripheral vacuole; a rod-shaped macronucleus; and a variable number of micronuclei, which are difficult to distinguish except by the Feulgen reaction. The two individuals of a double cyst (Fig. 6) share a common ectocyst, but each has its own endocyst. As to the mesocyst, the outer portion is shared in common, but the inner portion is continued over each, much like a separate membrane. The protoplast of each member of a double cyst shows the components already mentioned for a single cyst.

The sequence of events in the excystment process is the same, regardless of the excystment medium, though the speed of the response is affected by temperature and by the nature of the medium. The process may be studied to advantage by transferring resting cysts to 0.05 per cent lettuce infusion. Certain of the events which are pertinent to this investigation are shown in Figures 1–5. These are camera-lucida drawings of the same resting cyst, made at uniform mag-



FIGS. 1–6.—Figs. 1–5 show successive stages in the excystment of the same resting cyst of *T. magna*. Fig. 1, the cyst upon transfer to excystment medium. Fig. 2, formation of fluid vacuoles in cytoplasm and accompanying increase in size of cyst; body cilia differentiated; cytopharynx indicated. Fig. 3, fusion of many vacuoles to form a single large vacuole; maximum size of cyst now attained, with maximum pressure being exerted from within on cyst membranes. Fig. 4, rupture of ectocyst and mesocyst, leaving organism inclosed in endocyst. Fig. 5, stage of rapid swelling of endocyst, which soon ruptures and disappears, freeing the organism. Fig. 6 shows the double resting cyst of *T. magna*. (All figures made with camera lucida.)

nification, with the cyst contents represented in optical section.

The resting cyst (Fig. 1) shows neither cyclosis nor rotation of the protoplast. Cyclosis, seemingly disorganized at first, initiates the excystment process. Then small, transparent vacuoles appear in the cytoplasm. The vacuoles, which result from the imbibition of water, increase in size and number, and the entire cyst increases proportionately in diameter (Fig. 2). The body cilia differentiate, thus giving the cytoplasm a striated appearance; and the rudiment of the cytopharynx develops. The organism rotates constantly within its cyst membranes. The cyst continues to increase in diameter as new vacuoles form; and the membranes, being stretched from within, become measurably thinner. Many of the vacuoles coalesce to form a single, large hemispherical vacuole (Fig. 3). At this stage the cyst attains its maximal diameter and the rotation of the contents ceases temporarily. Suddenly, the ectocyst and mesocyst rupture and, being no longer under tension, contract and seemingly draw away from the organism (Fig. 4). Thus the ciliate, together with its endocyst, which now becomes clearly visible for the first time, is, in a sense, extruded from its outermost membranes. The endocyst increases greatly in diameter, while the imprisoned ciliate rapidly assumes its adult shape and struggles to escape (Fig. 5). Finally, the endocyst ruptures and apparently dissolves in the medium, thus freeing the ciliate.

At 20° C. the changes shown in Figures 1–4 usually occupy about 2¼ hours, whereas escape from the endocyst requires only 15 minutes. The cyst shown in Figures 1–5 had originally an over-all diameter of 114 μ. At the stage of the enlarged vacuole (Fig. 3) this dimension had increased to 136 μ. Just before the escape of the ciliate (Fig. 5) the endocyst measured 161 μ in diameter.

METHODS

Tillina magna was collected in late summer as dried cysts in debris from former pools in a meadow near Chapel Hill, North Carolina. The specimens which emerged when such debris was immersed in water were readily cultivable in a lettuce-salt medium to which loops of the bacterium *Pseudomonas fluorescens* were added as food. Details of the method of culture are given by Beers (1944).

All cysts used in the experiments were members of a single clone. The use of cysts from a pure line cultured under controlled conditions, while serving to eliminate in experimental work any variables arising from genetic diversities, may in itself introduce vitiating factors, should the progenitor of the line prove to be atypical. The present cysts were derived from Line A (Beers, 1944), a pure line which was cultured without loss of vitality and without the appearance of any structural or physiological abnormalities for 220 days, or 579 generations. All evidence indicates, therefore, that the cysts were quite normal and that the results may be accepted as typical for the species.

In order to conduct comparative excystment studies, it is evident that the experimental animals must encyst invariably under well-controlled and standardized conditions. Barker and Taylor (1933) and Johnson and Evans (1941) obtained standard cyst preparations of *Colpoda* and *Woodruffia*, respectively, by depriving the ciliates of food in balanced salt medium—a solution of inorganic salts of Ca, Na, K, and Mg. This method has special merit in that the medium is completely reproducible and does not support bacterial life. It is the method which I planned to use exclusively in this study, but unforeseen difficulties arose. The tillinas, when introduced into sterile balanced

medium or into any of nearly one hundred modifications of the formula, always failed to behave normally or to yield anything in the nature of consistent results with reference to encystment. All swam erratically at first, in small circles beneath the surface film; some burst and thereby altered the inorganic nature of the medium; some encysted, but after an extremely variable length of time; others swam for days and finally perished. Hence, the method was abandoned, and the following procedure was adopted. One cubic centimeter of sterile 0.05 per cent lettuce infusion, prepared by boiling 0.05 gm. of powdered dried lettuce (*Lactuca sativa capitata*) 5 minutes in 100 cc. distilled water, was put into a square watch glass (Columbia culture dish), and 10 large tillinas and 5 loops of *P. fluorescens* from a nutrient-agar plate were added. The culture was removed to a moist chamber in a constant-temperature room kept at 20° C. After a day, when about 50 tillinas were present, 5 more loops of bacteria were added, for *Tillina* is an avid feeder. At the end of another day the culture usually contained 400–450 tillinas, many of which were swimming actively in search of food; and on the third day it contained 500–750 resting cysts but no active specimens. After 4 more days, in which the cysts reached a state of maturity or physiological stability, they were ready for use in excystment experiments. Thus the standard cyst preparations were actually completion cultures which were prepared at regular intervals throughout the study. (It was readily demonstrated that encystment in such cultures was the result of depletion of the food supply. If additional loops of bacteria were added to the flourishing cultures—10 loops on the third day and a larger number on the fourth—reproduction continued, until as many as 5,000 individuals were present in each 1-cc. culture.) While these conditions of encystment are not as completely reproducible as those of a balanced salt-medium preparation, in that lettuce infusion may vary in composition and bacterial contaminants are not excluded, the cysts as a whole yielded remarkably consistent results and clearly possessed a high degree of uniformity. Periodic examinations of numbers of such cysts, which were suitably protected from evaporation of the medium, showed conclusively that they never became active spontaneously, and excystment tests showed that they remained viable for at least 8 months.

I am unable to explain satisfactorily the failure of *T. magna* to react normally and to encyst in solutions of inorganic salts. The evidence indicates that *Tillina* lacks the capacity for adjustment to chemical and physical changes which *Colpoda* and *Woodruffia* possess. In a sense, the failure of a ciliate to respond normally in an inorganic fluid should occasion no great surprise, for a ciliate like *Tillina* has probably never encountered in the course of its entire existence on earth a purely inorganic medium; its dependence on bacterial food restricts its habitat naturally to a medium rich in organic materials.

All of the experiments herein reported were performed at 20° C. in a constant-temperature room, and all concern the excystment-inducing activity of fluids. The method of testing the activity of a fluid was the following: Twenty cysts of age 4–10 days were transferred to 1 cc. of the test fluid contained in a watch glass and were washed gently by means of a pipette. After 2 minutes they were removed to a second watch glass of fluid and washed again. Finally, they were transferred to depression slides, 10 cysts being put into 0.2 cc. of test fluid in each depression, and the slides were removed to a moist chamber immediately. After the passage of 1.5 hours, a period in

which excystment was never observed to occur, the slides were examined under the dissecting binocular at 15-minute intervals for 2.5 hours, and records were made at such intervals of the progress of the excystment process in each cyst. Fluids which had considerable excysting capacity usually induced activation within 4 hours; fluids which proved to have little such capacity acted more slowly, and the observations after the fourth hour were made at less frequent intervals. Thus the time required for the activation of each cyst was determined with reasonable accuracy—often with an average error of observation of only 7.5 minutes.

All experiments were terminated after 20 hours, and fluids which were ineffective within this length of time were considered to be negative. Furthermore, some of the fluids were nutrient, and bacteria were developing in them in numbers after 20 hours. Their presence in the test fluids was objectionable, as constituting an undesirable experimental variable. Since it is practically impossible to render a living *Tillina* cyst bacteria-free, because of the sticky nature of its ectocyst, no attempt was made to exclude bacteria from the test slides. Their numbers were kept at a minimum through the use of initially sterile fluids, and in all crucial experiments the cysts became active long before the bacteria could possibly produce an effect.

In practice, the excysting capacity of eight fluids (e.g., eight concentrations of hay infusion) was investigated simultaneously, using 160 cysts in 16 groups of 10 each. On succeeding days the experiment was repeated, perhaps several times, until eventually data on a considerable number of cysts were available for each fluid. From these data the mean excystment time and the standard error of the mean were calculated for the cysts of each fluid. Two fluids were judged to possess significantly different excysting activity if the difference between their two means exceeded three times the larger standard error.

It was conclusively established at the outset that the mechanical agitation of the washing process does not of itself induce excystment. This was ascertained by subjecting hundreds of cysts to the mechanical operations of washing in the encystment medium and by distributing such cysts, in groups of 10, to test slides in encystment medium, quite as in the regular experiments. Not one of these cysts ever became active as a result of this treatment.

The practice of washing the cysts in the fluid to be tested was important as a means of removing the old encystment medium. In the absence of such washing, the cysts, upon transfer to a test fluid, may remain enveloped in a stratum of encystment medium, and ready access of the test fluid to the cyst membranes is denied.

Single cysts were used in all experiments, with the exception of Experiment 2. Any departures from the standard procedure, as outlined above, will be mentioned in the descriptive material which follows.

EFFECT OF DISTILLED WATER AND CERTAIN PLANT INFUSIONS ON EXCYSTMENT

Preliminary studies showed that a number of plant infusions were effective excysting agents, and it was assumed that excystment in *Tillina* resulted from the action of specific organic substances in such infusions. This assumption soon proved to be quite erroneous when it was discovered, by chance, that distilled water alone was an extremely effective excysting agent. This observation necessitated a precise evaluation of the effect of distilled water and led to the following comparative studies on the

relative effectiveness of distilled water and various plant infusions.

Expt. 1. Distilled water and 0.05 per cent lettuce infusion.—The water was triply distilled from an all-Pyrex system. The infusion was prepared as already described. Usually it was autoclaved and stored in 5-cc. amounts until needed, since autoclaving does not impair the excysting capacity of plant infusions. Only single cysts were used. The 300 cysts which were twice washed in distilled water and transferred, in 30 groups of 10 cysts each, to the test slides, in the manner already described, became active without exception and showed a mean excystment time of 2.87 hours (Table 1). Some specimens emerged after 2.25 hours; all were active at the end of 3.5 hours. Likewise, all of the cysts treated with lettuce infusion excysted, but with a mean excystment time of only 2.49 hours. The results show: (1) that both distilled water and 0.05 per cent lettuce infusion are effective excysting agents; (2) that excystment proceeds more rapidly in lettuce infusion; and (3) that the difference in the mean excystment times is a significant one.

Expt. 2. Distilled water and 0.05 per cent lettuce infusion.—This was essentially a repetition of Experiment 1, but with the difference that double resting cysts were used. To facilitate the counting of the excysted specimens, the cysts were tested in groups of only 5 to each 0.2-cc. volume of fluid. The mean excystment times (Table 1) were in close agreement with those of Experiment 1, and again there was a significant difference in the times. The 2 specimens of 1 double cyst failed to emerge in distilled water, and hence this group showed only 99 per cent excystment. However, this cannot be construed as significant, for in other experiments, as will be seen, a cyst sometimes failed to excyst in lettuce infusion.

Effect of a continuous stream of distilled water: Certain of my colleagues were frankly skeptical with reference to the excystment-inducing capacity of distilled water per se, maintaining that excystment under such conditions was actually induced by organic substances which were present in the cyst membranes and which diffused into the water in suitable dilute concentrations to produce excystment. They asked whether the cysts would become active if subjected to a continuous gentle stream of distilled water, which would serve to remove immediately any such substances from the vicinity of the cysts. The point seemed worthy of investigation. Accordingly, a piece of glass tubing having a length of 60 cm. and an inside diameter of 2.5 mm. was bent into such a shape that a portion of it might rest horizontally on the stage of the binocular, with one end continuing upward into a beaker of water and the other end extending downward into a dish at the side of the stage. Thus the tube might function as a siphon. The excurrent end was narrowed so that water could flow through it at the rate of 1 cc. per minute. At this rate of flow a column of water 20 cm. in length passed each cyst per minute. Twenty large, active tillinas and sufficient encystment medium to form a column 5 cm. in length were introduced into the empty tube and made to come to rest in the horizontal segment. Then the ends of the tube were sealed with paraffin, to hold the column in place and to prevent evaporation. In practice these operations involve no great difficulty, since the somewhat globose shape of a tillina permits it to be moved in a fluid column through any length of tubing without being trapped and flattened against the inner wall by the meniscus. Within a day the 20 tillinas produced 1 double cyst and 38 single cysts, all of which cemented themselves by the ectocyst to the inner

TABLE 1

EFFECT OF DISTILLED WATER AND CERTAIN PLANT INFUSIONS ON EXCYSTMENT

Expt. No.	No. of Cysts Tested	Treatment	Percentage of Excystment	Mean Excystment Time in Hours ± Standard Error	Range of Excystment Time in Hours
1.....	300	Distilled water	100.0	2.87±0.015	2.25– 3.5
	300	0.05% lettuce infusion	100.0	2.49±0.015	2.0 – 3.0
2.....	100	Distilled water	99.0	2.85±0.022	2.25– 3.75
	100	0.05% lettuce infusion	100.0	2.46±0.019	2.0 – 3.0
3.....	50	Distilled water	100.0	2.81±0.031	2.5 – 3.25
	50	0.025% lettuce infusion	100.0	2.72±0.033	2.25– 3.25
	50	0.05% lettuce infusion	100.0	2.42±0.031	2.25– 3.0
	50	0.075% lettuce infusion	100.0	2.73±0.038	2.25– 3.25
	100	0.1% lettuce infusion	100.0	3.05±0.044	2.5 – 4.0
	80	0.2% lettuce infusion	100.0	4.23±0.099	2.75– 6.0
	80	0.4% lettuce infusion	95.0	9.98±0.534	3.25–15.0
	80	0.5% lettuce infusion	96.3	13.93±0.265	7.0 –15.0
	80	0.75% lettuce infusion	93.8	14.00±0.157	10.0 –15.0
	80	1.0% lettuce infusion	97.5	15.0	.....
	80	1.5% lettuce infusion	46.3	15.0	.....
	50	2.0% lettuce infusion	14.0	15.0	.....
	50	3.0% lettuce infusion	0.0	.....	.....
4.....	100	Distilled water	99.0	2.92±0.027	2.75– 3.5
	100	0.025% timothy infusion	100.0	2.44±0.018	2.25– 2.75
	100	0.05% timothy infusion	100.0	2.43±0.016	2.0 – 2.75
	100	0.075% timothy infusion	100.0	2.53±0.022	2.0 – 2.75
	100	0.1% timothy infusion	100.0	2.80±0.043	2.25– 3.75
	100	0.2% timothy infusion	100.0	3.60±0.047	2.5 – 4.0
	80	0.3% timothy infusion	100.0	4.34±0.089	3.0 – 5.5
	80	0.4% timothy infusion	100.0	8.72±0.310	4.5 –10.0
	80	0.5% timothy infusion	100.0	10.61±0.359	6.0 –15.0
	80	0.75% timothy infusion	90.0	10.78±0.363	8.0 –15.0
	80	1.0% timothy infusion	95.0	10.03±0.383	7.0 –15.0
	80	2.0% timothy infusion	82.5	15.0	.....
	60	3.0% timothy infusion	63.3	15.0	.....
	50	4.0% timothy infusion	44.0	15.0	.....
	50	5.0% timothy infusion	0.0	.....	.....
5.....	50	Distilled water	100.0	2.89±0.035	2.5 – 3.25
	40	0.025% *Elodea* infusion	100.0	2.42±0.033	2.0 – 2.75
	100	0.05% *Elodea* infusion	100.0	2.35±0.022	2.0 – 2.75
	50	0.075% *Elodea* infusion	100.0	2.84±0.028	2.5 – 3.25
	100	0.1% *Elodea* infusion	100.0	2.99±0.065	2.25– 4.0
	50	0.2% *Elodea* infusion	96.0	5.72±0.342	3.5 –10.0
	40	0.3% *Elodea* infusion	80.0	15.0	.....
	40	0.4% *Elodea* infusion	20.0	15.0	.....
	40	0.5% *Elodea* infusion	0.0	.....	.....
6.....	50	Distilled water	100.0	2.89±0.032	2.5 – 3.25
	50	0.025% alfalfa infusion	100.0	2.37±0.023	2.0 – 2.5
	50	0.05% alfalfa infusion	100.0	2.23±0.016	2.0 – 2.5
	50	0.075% alfalfa infusion	98.0	2.37±0.034	2.0 – 2.75
	50	0.1% alfalfa infusion	100.0	2.49±0.031	2.0 – 2.75
	50	0.25% alfalfa infusion	100.0	4.33±0.067	3.5 – 5.0
	50	0.5% alfalfa infusion	100.0	6.86±0.167	5.0 – 8.0
	50	1.0% alfalfa infusion	92.0	7.39±0.089	6.0 – 8.0
	50	1.5% alfalfa infusion	30.0	8.07±0.274	7.0 –10.0
	50	2.0% alfalfa infusion	12.0	9.00±1.178	7.0 –15.0
	50	3.0% alfalfa infusion	0.0	.....	.....

wall of the tube. Five days later distilled water was siphoned continuously through the tube, and the condition of the cysts was observed and recorded at intervals. After 1.5 hours many of the cysts had increased appreciably in diameter, and the protoplast was rotating in each. After another hour a few individuals were escaping from their cyst membranes, and at the end of 3.5 hours all of the specimens had excysted. Some of them were recovered from the excurrent stream and cultured in lettuce infusion. Their structure and physiological activities were entirely normal. Two repetitions of the experiment, using 28 10-day-old cysts and 22 15-day-old cysts, respectively, gave results which agreed in all respects with those just described. Such results show conclusively that distilled water is in itself a powerful excysting agent and that excystment is not entirely dependent on the presence of specific organic compounds.

The reader may wonder about the behavior and survival of excysted specimens in distilled water. Such specimens, being small and lacking food inclusions, have considerable tolerance for distilled water, though large, well-fed specimens commonly burst upon transfer to it. In practice, the first specimens to emerge in the distilled water of a test culture usually swim sluggishly and sometimes are much vacuolated. Later these specimens, as well as those that follow them, display the normal, energetic, spiraling behavior of a ciliate in search of food. Actually, when 10 tillinas have excysted in 0.2 cc. distilled water, they have undoubtedly set free in the medium various by-products of metabolism, so that the medium may no longer be looked on as distilled water. These changes in the chemical composition of the medium, together with considerable tolerance for a hypotonic medium, insure a high percentage of survival.

Expt. 3. Lettuce infusion.—This experiment was designed to test the excystment-inducing activity of different concentrations of sterile lettuce infusion, all of which were prepared by boiling powdered dried lettuce 5 minutes in distilled water. Concentrations weaker than 0.1 per cent were prepared by diluting this concentration with distilled water; those between 0.1 and 1 per cent were made by diluting the latter; the higher concentrations were prepared individually. In order to obtain additional comparative data on the effect of water alone, some of the cysts of this experiment, and of succeeding ones as well, were tested, as formerly, with distilled water.

The difference between distilled water and the weakest infusion of Table 1 (0.025 per cent) was not significant. (Several weaker infusions were tested, but these also had the same effect as distilled water and are therefore omitted from the table.) The usual difference between water and 0.05 per cent was noted. The difference between 0.05 per cent and 0.075 per cent, while significant, was in the direction of a decrease in the rate of excystment; i.e., the tillinas in 0.075 per cent required a longer time for emergence. In fact, distilled water and 0.025 and 0.075 per cent lettuce infusions gave remarkably uniform results. These solutions induced the activation of every cyst in approximately the same mean time. In concentrations of 0.1–1.0 per cent the mean excystment time increased as the concentration increased, and with significant differences between successive increases in concentration. Furthermore, the individual cysts which were subjected to these concentrations showed far more variation in their excystment times, as the last column of Table 1 indicates. No observations were made during the night between the tenth and twentieth hours of an experiment. Hence,

the mean excystment time of 15 hours recorded for concentrations 1–2 per cent merely indicates that such specimens emerged between the tenth and twentieth hours. No specimens whatsoever excysted in 3 per cent infusion.

Not only did the mean excystment time increase in the infusions of higher concentration, but the percentage of excystment decreased. Only 95 per cent of the cysts were activated by 0.4 per cent infusion, fewer than half of them by 1.5 per cent, and none, as said, by 3 per cent. However, the cysts that failed to excyst were not actually killed by the infusions; when treated with distilled water or weak infusion they showed a high percentage of excystment.

Concentrations of 4 and 5 per cent were also tested, but with negative results, which are not included in Table 1. Many of the cysts were killed by a 20-hour exposure to these concentrations.

Expt. 4. Timothy infusions.—Since infusions of dried timothy (*Phleum pratense*) are known to possess a high degree of excysting activity, the effect of a series of sterile infusions of different concentrations was investigated, following the plan of Experiment 3. Stock infusions were prepared by boiling suitable amounts of chopped dried leaves and stems 10 minutes in distilled water, and certain of these were diluted to furnish weaker solutions, as in Experiment 3. The results are presented in Table 1 and need little explanation. The weakest three concentrations of the table (0.025, 0.05, and 0.075 per cent) induced excystment more rapidly than distilled water. (Several concentrations which were weaker than 0.025 per cent were also tested, but all of them agreed in activity with distilled water.) The concentration of 0.1 per cent did not differ significantly from distilled water. As the concentration increased, the mean excystment time increased, until finally in 5 per cent infusion, which killed many of the cysts, no specimens emerged.

Concentrations of 0.025–0.5 per cent induced 100 per cent excystment; higher concentrations produced a gradual decrease in the percentage of excystment. Thus the results paralleled in their essential features those obtained with lettuce infusion.

Expt. 5. Elodea infusions.—Johnson and Evans (1940) found that sterile infusions of elodea induced excystment in *Woodruffia;* and at their suggestion similar infusions, prepared in this study by boiling dried chopped leaves and stems of *E. canadensis* 5 minutes in distilled water, were tested on the cysts of *Tillina* (Table 1). The activity of two of the concentrations (0.025 and 0.05 per cent), as measured by the mean excystment time, exceeded that of distilled water, and that of the next two concentrations equaled that of distilled water, though all of these concentrations induced 100 per cent excystment. Higher concentrations lengthened the mean excystment time and decreased the percentage of excystment, which fell to zero in 0.5 per cent. Several higher concentrations, none of which killed the cysts, were tested, but with negative results. Concentrations weaker than 0.025 per cent had essentially the activity of distilled water.

Expt. 6. Alfalfa infusions.—These were prepared by boiling powdered, dried leaves of alfalfa (*Medicago sativa*) 5 minutes in distilled water. The results (Table 1) agreed faithfully as regards essentials with those of Experiments 3–5, in that certain of the more dilute infusions induced excystment more rapidly than distilled water, whereas the more concentrated solutions increased the mean excystment time and decreased the percentage of excystment.

Thus the results of Experiments 1–6 demonstrate with reference to *T. magna* that both distilled water and sterile plant

infusions are active excysting agents but that the excystment process is accelerated by the infusions. However, it is evident that the substances which are responsible for such acceleration are effective only when present at certain relatively low levels of concentration; when present in too great concentration, either they are ineffective in themselves or their potential effectiveness is suppressed by other chemical or physical properties of the infusion.

EFFECT OF SOME SALTS OF ORGANIC ACIDS AND DEXTROSE (SINGLY AND IN ADMIXTURE) ON EXCYSTMENT

The conclusive demonstration of Haagen-Smit and Thimann that salts of plant acids are active excysting agents for *Colpoda* cysts, particularly when accompanied by a carbohydrate cofactor, suggested an inquiry into the effect of such compounds on the cysts of *Tillina*. Unfortunately, I have had opportunity to test only dextrose and two organic salts, and no opportunity to investigate the summation effect. Nonetheless, the results are of interest (Table 2). In each experiment some cysts were tested simultaneously in distilled water, and in certain experiments some were tested in lettuce infusion, for comparative purposes. The remarkably consistent response of the cysts to distilled water and 0.05 per cent lettuce infusion throughout this entire study attests to the uniformity of the standard cyst preparations and validates the method of comparing the results of experiments performed at different times.

Expt. 7. Na acetate.—Two concentrations (0.01 and 0.02 per cent) induced excystment significantly more rapidly than distilled water, though the activity of these concentrations scarcely equaled that of the weaker plant infusions of Experiments 3–6 or that of lettuce infusion of this experiment. A concentration of 0.04 per cent had approximately the effectiveness of distilled water, whereas higher concentrations increased the mean excystment time, as well as the range, and decreased the percentage of excystment. The failure of one cyst each to become active in 0.02 and 0.04 per cent, thus giving a percentage of excystment of 98.8, cannot be considered to have significance. Thus the general effect of Na acetate was similar to that of plant infusions, though its activity was less intense.

Expt. 8. K citrate.—Of the solutions of K citrate tested, only the 0.005 per cent concentration exceeded distilled water in activity, though the 0.01 per cent concentration equaled it. Higher concentrations agreed in effect with similar concentrations of Na acetate and plant infusions.

Expt. 9. Dextrose.—Two concentrations (0.025 and 0.05 per cent) exceeded distilled water in activity, and four concentrations (0.075–0.5) equaled it. The remaining concentrations yielded results like those already described in Experiments 3–8. With reference to Na acetate, K citrate, and dextrose, any concentration which was appreciably weaker than the first concentration of Table 2 had the effect of distilled water.

Expts. 10 and 11. Acetate, citrate, and dextrose in admixture.—All of the combinations of these substances which were tested exceeded distilled water in activity, and all equaled the more effective concentrations of plant infusions. The acetate-dextrose mixtures exceeded the activity of acetate alone (Expt. 7) and of 0.05 per cent dextrose alone (Expt. 9). There is, then, some evidence of a cofactor effect; but it is inconclusive, in view of the effectiveness of 0.025 per cent dextrose. The first citrate-dextrose mixture of Experiment 11 was in no wise superior in its activity to either component (Expts. 8 and 9), though the second

mixture exceeded its individual components in activity. Again there is evidence of a cofactor effect at certain levels of concentration.

From Experiments 7–11 it is seen that certain concentrations of dextrose and salts of organic acids are nearly as effective as plant infusions in inducing excystment. While these findings are in essential agreement with the observations of Haagen-Smit and Thimann on *Colpoda*, further work is needed to demonstrate clearly in *Tillina* the summation and cofactor effects.

TABLE 2

EFFECT OF SOME SALTS OF ORGANIC ACIDS AND DEXTROSE (SINGLY AND IN ADMIXTURE) ON EXCYSTMENT

Expt. No.	No. of Cysts Tested	Treatment	Percentage of Excystment	Mean Excystment Time in Hours ± Standard Error	Range of Excystment Time in Hours
7.....	60	Distilled water	100.0	2.83±0.038	2.25– 3.25
	80	0.01% Na acetate	100.0	2.58±0.027	2.25– 3.0
	80	0.02% Na acetate	98.8	2.68±0.042	2.0 – 3.25
	80	0.04% Na acetate	98.8	2.77±0.055	2.25– 3.5
	80	0.05% Na acetate	77.5	3.40±0.128	2.25– 6.0
	80	0.075% Na acetate	56.3	4.05±0.285	2.25– 8.0
	80	0.1% Na acetate	46.3	3.68±0.142	2.5 – 5.0
	80	0.15% Na acetate	0.0	.....	.....
	80	0.05% lettuce infusion	100.0	2.51±0.026	2.25– 3.0
8.....	50	Distilled water	100.0	2.84±0.049	2.25– 3.25
	50	0.005% K citrate	98.0	2.45±0.032	2.25– 3.0
	50	0.01% K citrate	100.0	2.71±0.030	2.25– 3.0
	50	0.02% K citrate	86.0	2.91±0.044	2.5 – 3.5
	50	0.03% K citrate	36.0	3.72±0.165	3.0 – 5.0
	50	0.04% K citrate	14.0	5.57±0.187	5.0 – 6.0
	50	0.05% K citrate	4.0	8.0	.....
	50	0.075% K citrate	0.0	.....	.....
9.....	100	Distilled water	100.0	2.87±0.031	2.5 – 3.5
	100	0.025% dextrose	100.0	2.38±0.020	2.25– 3.0
	100	0.05% dextrose	100.0	2.58±0.027	2.25– 3.25
	100	0.075% dextrose	98.0	2.71±0.046	2.25– 3.5
	100	0.1% dextrose	100.0	2.81±0.037	2.25– 4.0
	100	0.25% dextrose	100.0	2.84±0.049	2.25– 3.75
	100	0.5% dextrose	100.0	2.83±0.060	2.25– 4.0
	100	0.75% dextrose	100.0	3.40±0.065	2.25– 4.0
	100	1% dextrose	88.0	3.94±0.116	2.5 – 6.0
	100	1.5% dextrose	28.0	5.41±0.421	3.5 –10.0
	100	2% dextrose	0.0	.....	.....
10.....	60	Distilled water	100.0	2.84±0.040	2.25– 3.25
	100	Equal parts 0.01% Na acetate and 0.025% dextrose	98.0	2.42±0.031	2.0 – 3.0
	100	Equal parts 0.02% Na acetate and 0.05% dextrose	100.0	2.44±0.028	2.0 – 3.0
	50	0.05% lettuce infusion	100.0	2.50±0.037	2.0 – 3.0
11.....	50	Distilled water	100.0	2.83±0.044	2.25– 3.25
	60	Equal parts 0.005% K citrate and 0.025% dextrose	100.0	2.47±0.031	2.0 – 2.75
	60	Equal parts 0.01% K citrate and 0.05% dextrose	100.0	2.48±0.033	2.0 – 3.0
	50	0.05% lettuce infusion	98.0	2.46±0.041	2.0 – 3.0

EFFECT OF YEAST EXTRACT AND SOME COMMON ORGANIC COMPOUNDS ON EXCYSTMENT

Expt. 12. Yeast extract (Difco Laboratories).—This substance was tested, since it was found to be a highly active excysting agent for *Colpoda* (Brown and Taylor, 1938) and a moderately active one for *Woodruffia* (Johnson and Evans, 1940). The various solutions, all of which were sterile when a test was begun, were prepared with distilled water and tested in the usual manner (Table 3). None of the solutions of yeast extract surpassed distilled water in excystment-inducing activity. Using the mean excystment time as the measure of activity, concentrations of 0.01–0.03 per cent were equivalent to distilled water, but higher concentrations were inferior. With reference to the percentage of excystment, concentrations of 0.01–0.075 per cent equaled distilled water, in that they induced approximately 100 per cent ex-

TABLE 3

EFFECT OF YEAST EXTRACT AND SOME COMMON ORGANIC COMPOUNDS ON EXCYSTMENT

Expt. No.	No. of Cysts Tested	Treatment	Percentage of Excystment	Mean Excystment Time in Hours ± Standard Error	Range of Excystment Time in Hours
12.....	80	Distilled water	98.8	2.86±0.035	2.5 – 3.25
	80	0.01% yeast extract	98.8	2.88±0.033	2.5 – 3.25
	80	0.02% yeast extract	100.0	2.82±0.035	2.5 – 3.5
	80	0.03% yeast extract	100.0	2.87±0.035	2.5 – 3.5
	80	0.05% yeast extract	100.0	3.12±0.036	2.75– 3.75
	80	0.075% yeast extract	100.0	3.20±0.051	2.75– 4.0
	80	0.1% yeast extract	87.5	3.40±0.034	3.0 – 4.0
	80	0.2% yeast extract	55.0	6.36±0.215	4.0 – 8.0
	80	0.3% yeast extract	30.0	8.67±0.304	6.0 –10.0
	80	0.5% yeast extract	0.0	.....	.....
13.....	80	Distilled water	100.0	2.84±0.033	2.5 – 3.25
	80	0.5% ethyl alcohol	100.0	2.82±0.031	2.5 – 3.25
	80	1% ethyl alcohol	100.0	2.81±0.029	2.5 – 3.25
	80	2% ethyl alcohol	100.0	2.86±0.026	2.5 – 3.25
	80	3% ethyl alcohol	90.0	3.33±0.051	2.75– 4.0
	80	4% ethyl alcohol	66.3	5.10±0.144	4.0 – 7.0
	80	5% ethyl alcohol	0.0	.....	.....
	80	0.05% lettuce infusion	100.0	2.41±0.023	2.0 – 2.75
14.....	80	Distilled water	100.0	2.86±0.023	2.5 – 3.25
	80	0.025% urea	100.0	2.82±0.024	2.5 – 3.25
	80	0.05% urea	97.5	2.88±0.024	2.5 – 3.25
	80	0.1% urea	100.0	2.98±0.023	2.5 – 3.25
	80	0.25% urea	90.0	3.08±0.034	2.75– 3.75
	80	0.5% urea	87.5	5.06±0.207	3.25– 8.0
	80	0.75% urea	72.5	5.75±0.214	3.75– 8.0
	80	1% urea	0.0	.....	.....
15.....	50	Distilled water	100.0	2.91±0.033	2.5 – 3.5
	50	0.025% glycine	100.0	2.36±0.033	2.0 – 2.75
	50	0.05% glycine	100.0	2.44±0.034	2.0 – 2.75
	50	0.075% glycine	100.0	2.53±0.046	2.0 – 3.0
	50	0.1% glycine	100.0	2.51±0.062	2.0 – 3.25
	50	0.25% glycine	100.0	2.65±0.068	2.25– 3.5
	50	0.5% glycine	88.0	3.18±0.116	2.25– 4.5
	50	0.75% glycine	78.0	6.07±0.474	3.0 –10.0
	50	1% glycine	32.0	5.72±0.428	4.5 –10.0
	50	1.5% glycine	0.0	.....	.....
	50	0.05% lettuce infusion	100.0	2.42±0.034	2.0 – 2.75

cystment; but higher concentrations induced activity in progressively fewer cysts.

Expt. 13. Ethyl alcohol.—The activity of two alcohols—ethyl and amyl—as examples of surface-active materials, was investigated on cysts of *Colpoda* by Barker and Taylor (1933), though with negative results. Nevertheless, an inquiry into the effect of an alcohol seemed advisable in the present study, and a series of test solutions was prepared by diluting 95 per cent ethyl alcohol with distilled water. Thus the solutions were prepared by volume, not by weight. After the usual two washings in the concentration to be tested, the cysts, together with 0.2 cc. of the test fluid, were introduced into a short piece of glass tubing, the ends of which were then sealed with paraffin. Thus the dilution of the fluids by evaporation of the alcohol was prevented; yet the excystment process could be observed through the transparent walls of the tubing. In general, the results, which are summarized in Table 3, agreed with those obtained with yeast extract. The weakest three concentrations had the same effect as distilled water; higher concentrations increased the mean excystment time and reduced the percentage of excystment.

Expt. 14. Urea.—The effect of urea, as an example of a waste product of nitrogen metabolism, was tested in the usual manner, using distilled water as the solvent. Its effect, summarized in Table 3, was similar to that of yeast extract and ethyl alcohol, in that only the most dilute concentrations equaled distilled water, whereas the higher concentrations either retarded or prevented excystment. With reference to yeast extract, ethyl alcohol, and urea, it is understood that concentrations below the first concentration of Table 3 had the same effect as distilled water.

Expt. 15. Glycine.—As the fourth and final organic substance of this series of experiments, glycine was tested, with results (Table 3) at variance with those obtained with yeast extract, alcohol, and urea but in agreement with those obtained with plant infusions, dextrose, and salts of organic acids. Glycine, in concentrations of 0.025–0.25 per cent (prepared, as usual, with distilled water), surpassed distilled water and equaled approximately the 0.05 per cent lettuce infusion in excystment-inducing capacity; higher concentrations retarded excystment.

EFFECT OF SOME INORGANIC SUBSTANCES AND OF DILUTION OF THE ORIGINAL ENCYSTMENT MEDIUM ON EXCYSTMENT

Expts. 16, 17, and 18. Salt medium, Na bicarbonate, Na sulphate.—The question arose as to whether aqueous solutions of nontoxic inorganic substances would induce excystment in *Tillina* and whether excystment, if induced by these substances, would proceed more rapidly or less rapidly than in distilled water.

To test this point, a series of different concentrations of the inorganic salt medium used by Barker and Taylor (1933), Johnson and Evans (1941), and others was prepared. The pH of each solution was established at 6.8 with a standard amount of KH_2PO_4—NaOH buffer mixture, and these differing concentrations were tested as usual. Then a series of unbuffered solutions of Na bicarbonate ($NaHCO_3$) and Na sulphate ($Na_2SO_4 \cdot 10H_2O$) was prepared and tested.

The results are summarized in Table 4, the data of which are largely self-explanatory. With reference to each of the three substances, the weakest two concentrations merely equaled distilled water, higher concentrations retarded or prevented excystment, and no concen-

tration surpassed distilled water in activity.

In practice, more concentrations were tested than are listed in Table 4. Of these, all between distilled water and the first concentration listed for each experiment agreed in effect with water alone, and concentrations higher than the last one of the table prevented excystment.

Expt. 19. Dilution of the original encystment medium.—That the mere dilution of the original encystment medium is sufficient to induce excystment was indicated in some of the exploratory

TABLE 4

EFFECT OF SOME INORGANIC SUBSTANCES AND OF DILUTION OF THE ORIGINAL ENCYSTMENT MEDIUM ON EXCYSTMENT

Expt. No.	No. of Cysts Tested	Treatment	Percentage of Excystment	Mean Excystment Time in Hours ± Standard Error	Range of Excystment Time in Hours
16.....	50	Distilled water	100.0	2.84±0.041	2.25– 3.25
	50	0.0025% salt medium	100.0	2.81±0.040	2.25– 3.25
	50	0.005% salt medium	98.0	2.84±0.052	2.25– 3.25
	50	0.01% salt medium	90.0	3.45±0.138	2.5 – 6.0
	50	0.02% salt medium	70.0	4.33±0.318	2.75– 8.0
	50	0.03% salt medium	78.0	5.18±0.228	3.0 – 8.0
	50	0.05% salt medium	34.0	7.47±0.540	3.0 –10.0
	50	0.06% salt medium	0.0	.....	.....
	50	0.05% lettuce infusion	98.0	2.37±0.027	2.0 – 2.75
17.....	50	Distilled water	100.0	2.87±0.035	2.25– 3.25
	40	0.01% Na bicarbonate	100.0	2.81±0.038	2.25– 3.25
	50	0.025% Na bicarbonate	98.0	2.85±0.063	2.5 – 3.5
	50	0.05% Na bicarbonate	78.0	3.28±0.117	2.5 – 5.0
	50	0.075% Na bicarbonate	38.0	4.82±0.271	3.0 – 6.0
	50	0.1% Na bicarbonate	0.0	.....	.....
18.....	50	Distilled water	100.0	2.82±0.034	2.25– 3.25
	50	0.01% Na sulphate	100.0	2.85±0.037	2.5 – 3.25
	50	0.02% Na sulphate	98.0	2.91±0.052	2.25– 3.5
	50	0.03% Na sulphate	90.0	3.03±0.052	2.5 – 3.5
	50	0.05% Na sulphate	88.0	3.01±0.055	2.5 – 3.75
	50	0.075% Na sulphate	74.0	3.36±0.066	2.75– 4.0
	50	0.1% Na sulphate	56.0	4.09±0.156	3.0 – 6.0
	50	0.2% Na sulphate	20.0	7.00	.....
	50	0.3% Na sulphate	0.0	.....	.....
	50	0.05% lettuce infusion	100.0	2.37±0.036	2.0 –2.75
19.....	80	8 parts 0.05% old lettuce infusion and 2 parts distilled water	32.5	7.38±0.373	6.0 –10.0
	80	6 parts 0.05% old lettuce infusion and 4 parts distilled water	71.3	5.57±0.291	4.0 –10.0
	80	5 parts 0.05% old lettuce infusion and 5 parts distilled water	82.5	5.53±0.319	3.5 –15.0
	80	4 parts 0.05% old lettuce infusion and 6 parts distilled water	78.8	5.73±0.379	3.75–15.0
	80	2 parts 0.05% old lettuce infusion and 8 parts distilled water	96.3	4.74±0.136	3.5 – 8.0
	80	0.05% old lettuce infusion	0.0	.....	.....
	80	0.1% old lettuce infusion	0.0	.....	.....
	80	0.2% old lettuce infusion	0.0	.....	.....
	80	Distilled water	100.0	2.92±0.030	2.5 – 3.5
	50	0.05% fresh lettuce infusion	100.0	2.51±0.040	2.0 – 3.0

work which preceded the formal experiments. A few drops of distilled water were added to several improperly stored completion cultures in order to compensate for loss of the medium through evaporation. Active tillinas were present on the following day. The significance of the excystment of these specimens was overlooked at the time; but in the light of later work it seemed likely that they excysted in response to the dilution of the medium, and a series of experiments was begun to test this possibility.

About thirty cultures were prepared, as usual with 0.05 per cent lettuce infusion, and allowed to go to completion. When they were a week old, suitable quantities of the encystment medium, which will be referred to as "old lettuce infusion," were removed, filtered, and variously diluted with distilled water, to give the five mixtures listed in Table 4. The two fluids were always thoroughly stirred together to give a uniform mixture. Not only were these dilutions of the encystment medium prepared, but two concentrates—0.1 and 0.2 per cent—were prepared by allowing suitable amounts of the encystment medium to evaporate to one-half and one-fourth of the original volume, respectively. The excysting activity of the foregoing seven fluids was then tested in accordance with the standard procedure.

The results are summarized in Table 4. The lowest dilution (eight parts old lettuce infusion to two parts water) induced the excystment of 32.5 per cent of the cysts with a mean excystment time of 7.38 hours. Six hours passed before the first specimen emerged. The following three dilutions (6:4, 5:5, and 4:6) gave essentially similar results, in that they induced the excystment of approximately 77 per cent of the cysts in 5.6 hours. The final dilution (2:8) induced 96.3 per cent excystment with a mean time of only 4.74 hours. In retrospect, it is unfortunate that dilutions 7:3 and 3:7 were not tested. Nevertheless, the results are decisive and the trend is clear; viz., dilution of the old encystment medium with distilled water suffices to induce excystment, and the percentage of excystment increases, whereas the mean excystment time decreases, with the degree of dilution. The next item of Table 4 (0.05 per cent old lettuce infusion) constitutes the control; the following two items represent the concentrates, neither of which induced excystment; the last two items attest to the normality of the cysts and supply additional comparative data.

DISCUSSION

The results obtained with distilled water show conclusively that organic materials are not necessary for excystment in *Tillina*. Excystment in distilled water is to be interpreted chiefly, if not exclusively, in terms of an environmental change of a physical nature. Distilled water, being unquestionably hypotonic to the cytoplasm, enters readily. In this process the plasma membrane acts as a semipermeable membrane; and perhaps the cyst membranes function in like manner, though little is known with certainty as to their permeability. There can be no doubt that water enters the cytoplasm in the early stages of excystment. The great increase in volume undergone by the protoplast and the obvious accumulation of large vacuoles in the cytoplasm attest to the entrance of water, which later is discharged by the contractile vacuole. The evidence indicates, therefore, that the primary excystment-inducing factor in *Tillina* is of an osmotic nature, involving the immersion of the cysts in a hypotonic medium and the consequent entrance of water into the protoplast.

As has been shown, extremely low concentrations of plant infusions have the same effect as distilled water. The excystment-inducing factor which is operative at these levels is therefore an osmotic one. However, the fact that cysts become active more rapidly in slightly higher concentrations shows that the osmotic factor is not the only one involved and indicates that such infusions contain effective concentrations of specific excystment-inducing substances. The question as to whether such substances merely alter the permeability of the plasma membrane and cyst membranes in such a way as to accelerate the entrance of water or whether they act directly on the protoplasm and thus stimulate it to differentiate more rapidly in the direction of the free-swimming condition awaits investigation. The assumption that specific excystment-inducing substances are present in fresh plant infusions necessitates the correlative assumption that such substances are absent in the lettuce infusion of the completion cultures. In reality, the two assumptions are not incompatible, for the metabolic activities of the bacteria and ciliates are sufficient to alter radically the chemical composition of the infusion of a completion culture. As still higher concentrations of plant infusions are employed, the percentage of excystment and the speed of the excystment process are decreased, as has been seen. These findings indicate that the accelerative effect of the specific substances is being offset by the increase in the concentration of the medium. Finally, a concentration is reached at which no excystment occurs. This concentration, it appears, is isosmotic with the cysts. Beyond it no further excystment occurs; and the cysts, as a matter of fact, become shrunken and die. It is clear, therefore, that the osmotic factor is constantly operative during excystment in plant infusions and that its positive effect may be intensified at certain levels of concentration by the presence of specific excystment-inducing substances.

The rapid excystment observed upon treatment of the cysts with certain dilute concentrations of Na acetate, K citrate, and dextrose indicates that these substances likewise have specific excystment-inducing properties. Since sugars and organic acids are known to occur in plant infusions, the evidence indicates that these substances, or more likely sugars and certain salts of the acids, are the active excystment-inducing principles of plant infusions, as the studies of Thimann and his associates indicate.

The failure of dilute solutions of yeast extract, ethyl alcohol, and urea to induce excystment more rapidly than distilled water leads only to the conclusion that these substances, with reference to the cysts of *Tillina*, lack specific excystment-inducing properties. The results obtained with various concentrations of each of these substances show that we are dealing solely with an osmotic effect.

The results obtained with glycine, dilute solutions of which exceeded distilled water in activity, may occasion mild surprise, since it is well known that amino acids penetrate cells slowly. However, this property is shared also by sugars and neutral salts of organic acids; yet these substances have been shown in both *Colpoda* and *Tillina* to have high excysting capacity. It seems likely that glycine, as well as plant infusions, dextrose, and salts of organic acids, alters the permeability of the cyst, thus allowing water to enter more readily, though further work is needed on the effects of amino acids before generalizations can be reached.

It has been shown that sufficiently dilute solutions of salt medium, Na bi-

carbonate, and Na sulphate induce excystment. In reality, we are dealing here primarily with an osmotic phenomenon, since higher concentrations suppress excystment, whereas lower concentrations induce it, and the percentage of excystment and speed of the response increase as distilled water is approached. Were the role of distilled water itself undetermined, specific excystment-inducing properties might be attributed erroneously to a great variety of chemical substances, both inorganic and organic.

Finally, the dilution of the original encystment medium remains to be considered. Again, the positive results of such dilution indicate that the effect is an osmotic one, which under natural conditions may be far more important than has been suspected. It is well known that in dry weather small pools, through evaporation and through the accumulation of metabolic waste, come to contain considerable amounts of organic and inorganic materials in solution. When such pools, which nearly always contain protozoan cysts, are flooded by gradual rainfall, the medium is appreciably diluted; and such dilution may constitute an important excystment-inducing factor.

SUMMARY

Resting cysts of *T. magna* were obtained by allowing lettuce-infusion cultures, which contained the bacterium *P. fluorescens* as food, to go to completion. Thus, encystment was the result of depletion of the food supply. Such cysts, which never excysted spontaneously, were then transferred to various fluids, the excystment-inducing capacity of which was under investigation.

Distilled water was found to have a high degree of excystment-inducing activity, in that it produced at 20° C. practically 100 per cent excystment, with a mean excystment time of slightly less than 3 hours. The results indicate that excystment under these conditions is induced by the hypotonicity of the water and that the primary excystment-inducing factor is of an osmotic nature.

Certain dilute concentrations of plant infusions induced 100 per cent excystment more rapidly than distilled water; concentrations weaker than these had the effect of distilled water; concentrations of increasing strength lengthened the mean excystment time and reduced the percentage of excystment, which finally fell to zero. The results indicate that plant infusions contain specific excystment-inducing substances which at certain levels of concentration have an accelerative effect when compared to distilled water and that the presence of such substances constitutes a secondary excystment-inducing factor.

Dextrose, Na acetate, K citrate, and glycine had the same effect as plant infusions, indicating that they, too, have specific excystment-inducing properties at certain levels of concentration.

Yeast extract, ethyl alcohol, urea, inorganic salt medium (a mixture of salts of Na, K, Ca, and Mg), Na bicarbonate, and Na sulphate in low concentrations induced excystment; but their activity never exceeded that of distilled water. Higher concentrations inhibited excystment. Hence, specific excystment-inducing properties cannot be attributed to these substances, their activity in lower concentrations being the result of the osmotic properties of these concentrations.

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NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

MITOSIS,[1] BY FRANZ SCHRADER

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FEW biological processes have received so much attention over so long a time by so many competent investigators with so few conclusive results as that of mitosis. It is a valuable service to bring together the ramifying literature in this field and to subject it to critical analysis as Professor Schrader has so ably done in his monograph. Dealing as it does with a process that plays a fundamental role in reproduction, growth, differentiation, and heredity, the work will appeal to a wide audience. Many readers will regret the size limitations of the volume and will wish for the fuller treatment which the author obviously could give.

The work deals with the mitotic movements of chromosomes and related phenomena, together with the mechanisms associated therewith. Cytoplasmic constituents are considered only so far as they are directly involved in the mitotic mechanism. Mitosis is revealed as a process of great complexity and one which is part of a larger cycle of chromosome maneuvers representing a "highly involved complex of processes" extending through the entire cell generation. In the light of these complexities one ceases to wonder why mitosis is still so little understood. Since the monograph is itself a summary, its contents can only be sampled in a review.

Following the Introduction (chap. i) the structure of the mitotic apparatus and its components is considered (chap. ii [34 pp.]), with the accompanying comment that "the incompleteness of our knowledge of such structure is . . . . in a sense a measure of our ignorance of the forces of mitosis." Whether the structural elements observed after fixation "exist as such also in living cells is not immediately at issue," but evidence reviewed leaves little doubt that most of them do. This includes spindle fibers, which "in some form or shape are a reality." Where centrioles are not discernible in either the living or the fixed condition, "the best working hypothesis would presume that . . . . the material basis for polarization is diffuse or at least more dispersed than in the orthodox spindle." "An element of fundamental importance in the movements of chromosomes," but one which has only recently "received the attention which it merits," is "the kinetochore or centromere" (point of spindle-fiber attachment on the chromosome). Observations and interpretations as to its struc-

[1] New York: Columbia University Press, 1944. Pp. 110.

ture differ so much, however, that any broad consideration "must at present stand largely on hypothetical grounds." The probable homology between the kinetochore and the astral centriole is brought out, and numerous illuminating departures from "orthodox" conditions are reviewed, including those involving multiple or diffuse kinetochores.

Recent evidence and the modern viewpoint, including that of the biochemist and biophysicist, are emphasized throughout the work. E.g., regarding asters the view is developed that "the astral ray represents a line of flow" in which "the long molecules of the ground substance are oriented in parallel rows so as to form fibrous configurations." Under spindle constituents: the continuous fiber is regarded as similar in structure and origin to the astral ray; the chromosomal fiber "is based on the kinetochore [and] has naturally been regarded as the spindle element most intimately involved in chromosomal movement"; interzonal connections are apparently of various types and origins.

Chaper iii, of 36 pages, is entitled "Hypotheses of Mitosis" and deals primarily with the mechanics of chromosome movement. Two main questions are posed (each involving a complex of minor ones): "(1) How is the metaphase established? (2) What brings about the movement of the chromosomes to the poles?" "It is indicative of our ignorance that we usually cannot see the bearing that the problems of prophase and interphase have on the points that puzzle us in metaphase and anaphase." The possibility that the chromosomes are pulled to the poles by contraction of spindle fibers is supported by evidence and arguments of several investigators, and comparison is made with the contraction of myofibrils. From the latter, "serious consideration might be accorded the suggestion that these two [contractions] involve fundamentally the same mechanism—namely, a change in the shape of a protein chain. . . ." Of greater merit are hypotheses involving two or more activities. Such hypotheses are critically evaluated, with the conclusion that those which "utilize a contraction and expansion of spindle constituents are confronted with many difficulties. Nevertheless, they are once more entering our considerations, and . . . . may soon alter the pessimistic attitude of the recent past" because of the newer concepts of the changes in form of protein chains.

The complexities and difficulties inherent in the problem of mitosis are well brought out by the author's discussion of views attributing chromosome movement to changes in viscosity or hydration of protoplasmic materials. Under "Electrostatics," after careful consideration of views and evidence, the conclusion is reached that: "No other forces furnish such a good explanation of the minor as well as the more obvious events of mitosis. But, by the same token, no other hypothetical consideration makes such a glaring exposure of our ignorance of the physical chemistry of the cell, for almost every step in such reasoning involves one or more assumptions."

The possible role of diffusion currents and of regional currents or lines of flow in the spindle is considered, and the reviewer is pleased to see that the often neglected hydrodynamic forces are given equal consideration with others as possibly affecting chromosome movements. Recent work on tactoids offers interesting possibilities; but, although "there is no question that we are concerned here with forces that may be extremely important in the mechanics of the cell . . . . , the difficulty lies in the fact that at present the physicist himself hardly knows enough about them to be able to suggest

anything like an exact application to such complicated biological phenomena as mitosis."

Special attention is given to the possibility that "the mitotic movements of chromosomes are largely autonomous" and that "the forces involved reside or originate in the chromosomes themselves"—a view which has acquired some prominence "through the study of special cases which rule out any explanation based on contraction, expansion, streaming or electrical forces" and also receives support from earlier experimental evidence.

Chaper iv (9 pp.) is devoted to a "survey of problems that are not ordinarily considered as entering into mitotic questions" and "that at present have no satisfactory answer," but which, nevertheless, bear directly or indirectly on the present subject. The work closes with a three-page conclusion in which the in adequacy of all current hypotheses i balanced against the fact that probabl the majority of hypotheses "have a po tion of truth in them." The inherent di ficulties in the experimental method attack are cited, and promising lines future investigation are depicted. Th latter section tends to lend a final chee ful and encouraging tone to a surve which, as the author says, might we seem disheartening. A very valuab bibliography of eighteen pages is ap pended.

Naturally, in such a concentrated a count, the author has had to pick an choose his material and omit a good de that he would have liked to include. I would be difficult to find fault with h selections.